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(71) Applicants:
- **Riken**
  Wako-shi, Saitama 351-0198 (JP)
- **Mikoshiba, Katsuhiko**
  Tokyo 181-0001 (JP)

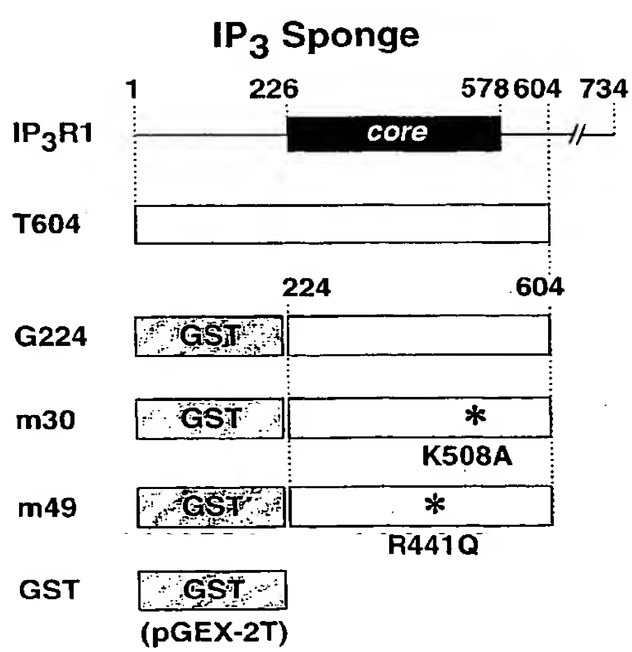
(72) Inventors:
- **Mikoshiba, Katsuhiko**
  Mitaka-shi, Tokyo 181-0001 (JP)
- **Furuichi, Teiichi**
  Chiba-shi, Chiba 263-0022 (JP)
- **Yoshikawa, Fumio**
  Yokohama-shi, Kanagawa 234-0054 (JP)
- **Uchiyama, Tsuyoshi**
  Shinagawa-ku, Tokyo 141-0021 (JP)

(74) Representative: **Maschio, Antonio et al**
D Young & Co,
21 New Fetter Lane
London EC4A 1DA (GB)

(54) **High affinity IP3-binding polypeptide**

(57) The present invention provides a high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate, to a gene encoding the polypeptide, to a recombinant vector including the gene, to a transformant including the vector and to a method for producing the high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate.

FIG. 1



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Description

FIELD OF THE INVENTION

[0001] The present invention relates to a high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate, to a gene encoding the polypeptide, to a recombinant vector including the gene, to a transformant including the vector and to a method for producing the high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate.

BACKGROUND OF THE INVENTION

[0002] Inositol 1,4,5-trisphosphate (hereinafter, also referred to as "IP_3") is one of second messengers which are produced by inositol phospholipid metabolism activated in response to an extracellular stimului such as hormones, growth factors, neurotransmitters or the like. IP_3 is a substance that induces the increase of intracellular calcium concentration. The IP_3-induced calcium increase is a crucial and highly universal signal transmission mechanism that is involved in many cell functions in a wide variety of animals. For example, IP_3 controls many physiological functions such as fertilization, blastogenesis, development and differentiation, cell growth, secretion, immune system, muscle contraction, and cranial nerve functions (gustation, vision, memory, learning, etc.) in diverse organisms, for example, invertebrata such as nematoda (nemathelminthes), Drosophila (arthropoda) and cuttlefish (mollusca), and vertebrata such as mouse and human.

[0003] On the molecular level, this mechanism is initiated by the binding between an IP_3 and its target, an IP_3 receptor. Specifically, when the IP_3 binds to the IP_3 receptor (a calcium channel susceptible to IP_3) present in an intracellular calcium-storing site (endoplasmic reticulum, etc.), the channel opens and releases calcium from the calcium-storing site into the cytoplasm, thereby controlling the activities of calcium-dependent proteins and enzymes.

[0004] Heparin, adenophostin (a kind of fungal metabolite) and Xestospongin (a kind of sponge metablite) are examples of substances that might affect the signal transmission by the IP_3-induced calcium. However, although heparin inhibits the binding between the IP_3 and the IP_3 receptor, its specificity is low since there are various targets in the cell. Adenophostin is an antagonistic agonist of the binding between the IP_3 and the IP_3 receptor, and is a powerful activator of the IP_3 receptor channel. However, its use is limited since its yield from fungus is low and it cannot transport across the membrane. Xestospongin has recently been reported as an inhibitor of the IP_3 receptor channel that does not influence the binding of IP_3. Again, its yield is low and there are still questions remaining as to its specificity. Thus, currently, there is almost no substance that is considered to effectively act on IP_3-induced calcium signal transmission. In particular, there has been no substance or system that inhibits IP_3-induced calcium signal transmission by specifically trapping IP_3 that has increased on the cell level.

SUMMARY OF THE INVENTION

[0005] The present invention provides a high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate, a gene encoding the polypeptide, a recombinant vector containing the gene, a transformant containing the vector and a method for producing the high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate.

[0006] In order to solve the above-described problem, the present inventors have gone through intensive studies and have succeeded in isolating a high affinity polypeptide having an extremely high binding activity to IP_3 from a protein including a part of the N-terminal amino acid region of an IP_3 receptor.

[0007] The present invention provides a recombinant polypeptide of the following (a), (b) or (c):

(a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO: 2;
(b) a polypeptide comprising an amino acid sequence having deletion, substitution or addition of at least one amino acid in the amino acid sequence shown in SEQ ID NO: 2, and having a high affinity binding activity to inositol 1,4,5-trisphosphate; or
(c) a polypeptide having at least 70% homology with the amino acid sequence shown in SEQ ID NO: 2, and having a high affinity binding activity with inositol 1,4,5-trisphosphate.

[0008] The present invention also provides a gene coding for a polypeptide of the above (a), (b) or (c); or a gene coding for a polypeptide having at least 70% homology with the gene and having a high affinity binding activity with inositol 1,4,5-trisphosphate.

[0009] The present invention further provides a gene comprising DNA of the following (d) or (e):

(d) DNA of a nucleotide sequence shown in SEQ ID NO: 1; or
(e) DNA of a nucleotide sequence having at least 70% homology with the DNA of the nucleotide sequence shown

in SEQ ID NO: 1, and coding for a polypeptide having a high affinity binding activity with inositol 1,4,5-trisphosphate.

[0010] The present invention provides a recombinant vector comprising any one of the above-described genes.

[0011] The present invention also provides a transformant comprising the above recombinant vector.

[0012] The present invention further provides a method for producing any one of the above-mentioned polypeptides, the method comprising: culturing the above-mentioned transformant; and collecting, from the obtained culture, a polypeptide having a high affinity binding activity to inositol 1,4,5-trisphosphate.

[0013] This and other advantages of the present invention will become apparent to those skilled in the art upon reading and understanding the following detailed description with reference to the accompanying figures.

[0014] This specification includes part or all of the contents as disclosed in the specification and/or drawings of Japanese Patent Application No. 10-242207 which is a priority document of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015]

Fig. 1 shows the structures of IP_3 sponges;

Figs. 2A-2C show high expression and IP_3-binding activity of T604;

Figs. 3A-3C are graphs showing the IP_3-binding activities of the IP_3 sponges;

Fig. 4 is a graph showing a curve of IP_3-binding inhibition depending on the IP_3 sponge concentration;

Figs. 5A-5F are graphs showing the effects of low-affinity G224-m30 and GST on IP_3-induced Ca^{2+} release;

Figs. 6A-6G are graphs showing the effect of high affinity IP_3 sponge G224 on inhibition of IP_3-induced Ca^{2+} release; and

Fig. 7 is a plot diagram showing an IP_3-induced Ca^{2+} release depending on the concentration of the high affinity IP_3 sponge G224.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0016] Hereinafter, the present invention will be described in more detail.

[0017] A polypeptide of the present invention specifically binds to IP_3 with a very high affinity, and includes a part (a cut) of N-terminal amino acid region of the natural IP_3 receptor (thus also referred to as a cut-type polypeptide). The polypeptide of the invention is often referred to as a high affinity IP_3-binding polypeptide.

1. Cloning a gene coding for the IP_3 receptor

[0018] In order to obtain a high affinity IP_3-binding polypeptide of the invention, a gene encoding the natural IP_3 receptor protein is cloned. The nucleotide sequence of the IP_3 receptor gene is already known (*Nucleic Acid Res*, 17: 5385-5386, 1989; *Nature* 342:32-38, 1989). The gene may, for example, be prepared according to the following gene engineering procedure.

(i) Preparation and screening of cDNA library encoding the IP_3 receptor

[0019] A known procedure may be employed to prepare mRNA of the IP_3 receptor. For example, total RNA is obtained by treating a tissue or a cell from a mouse brain with a guanidine reagent, a phenol reagent or the like. Then, poly(A) +RNA(mRNA) is obtained according to an affinity column method or a batch method using poly (U) sepharose, etc. By using the obtained mRNA as a template as well as oligo dT primer and reverse transcriptase, a single-stranded cDNA is synthesized. Based on the single-stranded cDNA, a doublestranded cDNA is synthesized and introduced into a suitable cloning vector to prepare a recombinant vector to transform *E.coli* or the like. The transformant is selected based on indices such as tetracycline and ampicillin resistance, thereby obtaining a cDNA library.

[0020] The transformation of *E.coli* may be conducted according to the method of Hanahan [Hanahan, D., *J. Mol. Biol.* 166:557-580 (1983)]. Specifically, the recombinant vector is added to a prepared competent cell under the presence of calcium chloride, magnesium chloride or rubidium chloride. When a plasmid is used as the vector, it should

contain a gene resistant to drugs such as tetracycline and ampicillin. Besides plasmids, a cloning vector such as λ phage may also be used.

[0021] The thus-obtained transformant is screened for strains with the DNA of interest by, for example, "expression cloning" through immunoscreening using an antibody, or by polymerase chain reaction (PCR) using a primer synthesized from a known sequence.

[0022] The thus-obtained DNA fragment or DNA amplified fragment coding for the antibody epitope is labeled with ^{32}P, ^{35}S, biotin or the like to be used as a probe for hybridizing with the transformant DNA denatured and bound on a nitrocellrose filter. Then, the obtained positive strains may be screened for the target DNA fragment.

(ii) Determination of the nucleotide sequence

[0023] The obtained clone is determined for its nucleotide sequence. The nucleotide sequence may be determined according to a known method such as Maxam-Gilbert chemical modification method, dideoxynucleotide chain termination method using M13 phage. Generally, the sequence is determined using an automatic DNA sequencer (e.g., Perkin-Elmer 373A DNA sequencer).

[0024] The nucleotide sequence of the natural (full-length) gene coding for the IP_3 receptor and the full-length amino acid sequence of the IP_3 receptor are shown in SEQ ID NOS. 3 and 4, respectively.

2. Design and synthesis of a gene coding for a high affinity IP_3-binding polypeptide of the invention

(i) Design and synthesis of a gene coding for a high affinity IP_3-binding polypeptide

[0025] A high affinity IP_3-binding polypeptide of the invention includes a cut of N-terminal amino acid region, that is, Amino acids 579 to at least 800, preferably Amino acids 579 to at least 734, of the amino acid sequence of the full-length IP_3 receptor protein (SEQ ID NO:4). According to the present invention, this cut-type polypeptide is also referred to as an IP_3 sponge (Fig. 1). Due to this cut, the polypeptide (IP_3 sponge) of the invention gains a very strong specific binding ability to IP_3 (high affinity IP_3-binding activity).

[0026] Herein, the phrase "high affinity" is used in the situation where the IP_3 sponge has an IP_3 affinity that is about 100 to 1,000 times (preferably 500 to 1,000 times) higher than that of the natural IP_3 receptor.

[0027] According to the present invention, the IP_3 sponge also includes at least the amino acid sequence shown in SEQ ID NO: 2, which corresponds to Amino acids 226-578 of the amino acid sequence of SEQ ID NO: 4. Herein, this region is referred to as a "core" region.

[0028] Based on the above-described facts, the length of the fragment of the invention and the length of the DNA coding for the fragment can be determined at ones discretion providing that the high affinity IP_3-binding activity is maintained. The fragment may include, for example, Amino acids 224-604 of the amino acid sequence of SEQ ID NO: 4 (encoded by Nucleotides 670-1812 of the nucleotide sequence of SEQ ID NO: 3); Amino acids 1-604 of the amino acid sequence of SEQ ID NO: 4 (encoded by Nucleotides 1-1812 of the nucleotide sequence of SEQ ID NO: 3); or Amino acids 1-734 of the amino acid sequence of SEQ ID NO: 4 (encoded by Nucleotides 1-2200 of the nucleotide sequence of SEQ ID NO: 3).

[0029] These fragments are obtained through PCR using primers that are designed based on nucleotide regions of the nucleotides shown in SEQ ID NO. 3 outside the regions of the respective fragments, as well as the DNA coding for the natural IP_3 receptor (SEQ ID NO: 3, *Nucleic Acid Res.* 17: 5385-5386, 1989; *Nature* 342: 32-38, 1989) as a template.

(ii) Preparation of a gene encoding a mutant-type IP_3 sponge of the invention (mutant-type IP_3 gene)

[0030] According to the present invention, the amino acid sequence of the IP_3 sponge may, at least partially, be introduced with a mutation. Such a mutant-type IP_3 sponge is also contemplated as the IP_3 sponge of the present invention. A mutation is introduced into the amino acid sequence, by mutating the nucleotide sequence of the gene coding for the amino acid sequence of the IP_3 sponge.

[0031] The mutation is introduced into the gene according to a known method such as Kunkel method, Gapped duplex method or any method equivalent thereof. For example, site-directed mutatagenesis may be employed in which a mutant oligonucleotide is used as a primer (Yoshikawa, F. et al., *J. Biol. Chem.* 271: 18277-18284, 1996). Alternatively, a mutation may be introduced by using a mutagenesis kit such as Mutant-K (Takara), Mutant-G (Takara) and a series of LA PCR *in vitro* Mutagenesis kits (Takara).

[0032] First, based on the nucleotides of the gene coding for the IP_3 sponge of the invention (also referred to as an "IP_3 sponge gene"), a primer is synthesized such that the primer includes a mutated nucleotide or site and about 10 nucleotides flanking the mutated nucleotide or site. Using this primer as well as the IP_3 sponge gene as a template,

PCR reaction is conducted. The resultant is purified and then treated with a suitable restriction enzyme, thereby obtaining the mutant-type IP_3 sponge gene of interest.

(iii) Determination of the nucleotide sequences

[0033] The nucleotide sequence of the genes obtained through (i) and (ii) is determined. The determination is conducted by a known method such as Maxam-Gilbert chemical modification method, dideoxynucleotide chain termination method using M13 phage, or any other method. Generally, an automatic sequencer (e.g., 373A DNA sequencer produced by Perkin-Elmer) is used.

[0034] A nucleotide sequence of an IP_3 sponge gene of the invention and an amino acid sequence of the IP_3 sponge of the invention are shown in SEQ ID NOS: 1 and 2, respectively. The polypeptide of this amino acid sequence may include at least one deletion, substitution, addition or the like as long as it has a high affinity with IP_3 and has an activity of specifically binding to IP_3.

[0035] For example, at least one, preferably about 1 to 10, more preferably 1 to 5 of the amino acids in the core region (the amino acid sequence shown in SEQ ID NO: 2) may be deleted; at least one, preferably about 1 to 10, more preferably 1 to 5 amino acids may be added to the amino acid sequence of the core region; or at least one, preferably 1 to 10, more preferably 1 to 5 of the amino acids in the core region may be replaced with other amino acids.

[0036] The polypeptide of the present invention is not limited by the length of the amino acid sequence as long as the amino acid sequence contains the amino acid sequence of the core region, and a cut of N-terminal Amino acids 579 to at lease 800, preferably N-terminal Amino acids 579 to at least 734 of the natural-type IP_3 receptor (SEQ ID NO: 4). For example, Amino acids 224-604 (polypeptide "G224") of the amino acid sequence shown in SEQ ID NO: 4, and the gene encoding G224 are also contemplated as the IP_3 sponge and the IP_3 sponge gene of the invention, respectively.

[0037] The polypeptide G224 may have a mutation of at least one, preferably about 1 to 10, more preferably 1 to 5 amino acids. Thus, the IP_3 sponge of the invention may include an amino acid sequence where Lysine at Position 508 of the amino acid sequence G244 is replaced with alanine (mutation "m30") or where arginine at Position 441 of the amino acid sequence G244 is replaced with glutamin (mutation "m49") (Figure 1). Herein, the numbers indicating the positions of the amino acids are based on the amino acid sequence shown in SEQ ID NO: 4 (e.g., Position 1 is the first amino acid of SEQ ID NO: 4).

[0038] A polypeptide including an amino acid sequence having 70% or more homology with the core region (SEQ ID NO: 2), and having a high affinity binding activity with inositol 1,4,5-trisphosphate is also contemplated as the present invention.

[0039] Also contemplated as the present invention is a gene coding for the polypeptide having the above-described mutation in its amino acid sequence, and having a high affinity binding activity with IP_3 receptor. In addition, a nucleotide sequence coding for the amino acids included in the IP_3 sponge of the present invention, and a degenerate isomer coding for the same polypeptide with different degenerate codons are also contemplated as the genes of the invention. Also contemplated as the present invention is DNA having at least 70% homology with the nucleotide sequence of these genes, for example, DNA of other type belonging to the IP_3 receptor gene family that codes for a region corresponding to the polypeptide of the present invention.

[0040] Once the nucleotide sequence of the gene of the present invention is determined, the gene may be obtained by PCR using a primer that is synthesized chemically or that is synthesized from the determined nucleotide sequence.

3. Preparation of recombinant vector and transformant containing IP_3 sponge gene of the invention

(i) Preparation of recombinant vector

[0041] A recombinant vector of the invention may be obtained by ligating (inserting) the IP_3 sponge gene of the invention to (into) a suitable vector. The vector for inserting the gene of the invention is not limited to a specific one as long as it is replicable in a host cell. Examples of such vector include but not limited to plasmid DNA and phage DNA.

[0042] The plasmid DNA is, for example, plasmid from *E.coli* (e.g., pET-3a, pBR322, pBR325, pUC118, pUC119, etc.), plasmid from bacillus (e.g., pUB110, pTP5, etc.), or plasmid from yeast (e.g., YEpl3, YEp24, YCp50, etc.). The phage DNA is, for example, λ phage. Similarly, an animal virus vector such as retrovirus, adenovirus or vaccinia virus vectors, or an insect virus vector such as a baculovirus vector may also be used. A fusion plasmid in which GST, GFP, His-tag, Myc-tag or the like is linked with each other may also be used (e.g., pGEX-2T, pEGFP-N3).

[0043] To insert the gene of the invention into the vector, first, the purified DNA is cleaved with suitable restriction enzymes. Then, the cleaved fragment is inserted into a restriction site or a multicloning site of the suitable vector DNA.

[0044] The gene of the present invention should be integrated into the vector such that the gene can function. If desired, the vector of the invention may include, other than the gene of the invention and the promoter, for example,

a cis-element (e.g., an enhancer), a splicing signal, a poly(A) tail signal, a selective marker, and a ribosome binding sequence (SD sequence) . Examples of the selective marker include a dihydrofolate reductase gene, an ampicillin-resistant gene and a neomycin-resistant gene.

(ii) Preparation of transformant

[0045] A transformant of the invention may be obtained by introducing the recombinant vector of the invention into a host cell in such a manner that the gene of interest is capable to be expressed. The host cell is not limited to a specific one as long as it can express the gene of the present invention. Bacteria such as genus *Escherichia* (e.g., *Escherichia coli*), genus *Bacillus* (e.g., *Bacillus subtilis*), genus *Pseudomonas* (e.g,. *Pseudomonas putida*), yeast such as *Saccharomyces* cerevisiae and *Schizosaccharomyces pombe,* animal cells (e.g., COS, CHO, HEK293, PC12 cells), and insect cells (e.g., Sf9 and Sf21) are exemplified.

[0046] When a bacterium such as *E.coli* is used as the host, it is preferable that the recombinant vector of the present invention is capable of autonomous replication in the host and that it includes a promoter, a ribosome binding sequence, the gene of the invention and a transcription termination sequence. The recombinant vector may also include a gene for controlling the promoter.

[0047] As the *E.coli, E. coli* BL21, JM109 and HB101 are exemplified and as bacillus, *Bacillus subtilis* MI 114 and 207-21 are exemplified.

[0048] Any promoter may be used as long as it can be expressed in a host cell like *E.coli,* For example, a promoter derived from *E.coli* or phage, e.g., trp promoter, lac promoter, p_L promoter or p_R promoter, may be used. Artificially designed and modified promoter like tac promoter may also be used.

[0049] The recombinant vector may be introduced into the host bacterium according to any method for introducing DNA into a bacterium. For example, calcium ion method (Cohen, S.N. et al., *Proc. Natl. Acad. Sci.*, USA, 69: 2110-2114 (1972)) and an electroporation method may be employed.

[0050] An yeast such as *Saccharomyces cerevisiae, Schizosaccharomyces pombe* or *Pichia pastoris* may also be used as the host. In this case, the promoter may be any promoter that can be expressed in the yeast. Examples of such promoter include but not limited to gal1 promoter, gal10 promoter, heat shock protein promoter, MF 1 promoter, PHO5 promoter, PGK promoter, GAP promoter, ADH promoter and AOX1 promoter.

[0051] The recombinant vector may be introduced into the yeast by any method for introducing DNA into an yeast. For example, electroporation method (Becker, D.M. et al., *Methods Enzymol.,* 194, 182-187 (1990)), spheroplast method (Hinnen, A. et al., *Proc. Natl. Acad. Sci.,* USA, 75, 1929-1933 (1978)), or lithium acetate method (Itoh, H., *J. Bacteriol.,* 153, 163-168 (1983)) may be employed.

[0052] An animal cell such as simian cell (e.g., COS-7, Vero), Chinese hamster ovary cell (CHO cell), mouse L cell, rat cell (e.g., GH3, PC12 or NG108-15) or human cell (e.g., FL, HEK293, HeLa or Jurkat) may also be used as the host. As a promoter, for example, SR promoter, SV40 promoter, LTR promoter or β-actin promoter may be used. Other than these promoters, an early gene promoter of human cytomegalovirus may also be used.

[0053] The recombinant vector may be introduced into the animal cell, for example, by an electroporation method, a calcium phosphate method or a lipofection method.

[0054] An insect cell such as Sf9 cell, Sf21 cell or the like may also be used as the host. The recombinant vector may be introduced into the insect cell, for example, by a calcium phosphate method, a lipofection method or an electroporation method.

4. Production of IP_3 sponge

[0055] The IP_3 sponge of the present invention may be obtained by culturing the above-described transformant, and recovering the IP_3 sponge from the culture product. The term "culture" as used herein refers to a culture supernatant, a cultured cell or microbial cell, or a cell or microbial cell debris.

[0056] The transformant of the invention is cultured according to a general method employed for culturing the host.

[0057] A medium for culturing the transformant obtained from a microorganism host such as *E.coli* or yeast may be either a natural or a synthetic medium providing that it contains carbon sources, nitrogen sources, inorganic salts and the like assimilable by the microorganism, and that it can efficiently culture the transformant.

[0058] As carbon sources, carbohydrate such as glucose, fructose, sucrose, starch; organic acids such as acetic acid, propionic acid; and alcohols such as ethanol and propanol may be used.

[0059] As nitrogen sources, ammonia; ammonium salts of inorganic or organic acids such as ammonium chloride, ammonium sulfate, ammonium acetate and ammonium phosphate; other nitrogen-containing compounds; Peptone; meat extract; corn steep liquor and the like may be used.

[0060] As inorganic substances, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, iron(II) sulfate, manganese sulfate, copper sulfate, calcium carbonate

and the like may be used.

[0061] The cultivation is generally performed under aerobic conditions such as shaking or aeration agitating conditions at 37°C for 6 to 24 hours. During the cultivation, pH is maintained at 7.0 to 7.5. pH is regulated with an inorganic or organic acid, an alkali solution or the like. If necessary, an antibiotic such as ampicillin, tetracycline or the like may be added to the medium during the cultivation.

[0062] When culturing a microorganism transformed with an expression vector using an inducible promoter, an inducer may be added to the médium at need. For example, isopropyl 1-thio-β-D-galactoside (IPTG) may be added to the medium when culturing a microorganism transformed with an expression vector pET-3a having T7 promoter (that is inducible with IPTG). When culturing a microorganism transformed with an expression vector using trp promoter (that is inducible with indole acetic acid (IAA)), IAA may be added to the medium.

[0063] A transformant obtained with an animal cell host may be cultured in a generally used medium such as RPMI1640 medium or DMEM medium, or a medium obtained by supplementing the generally used medium with fetal bovine serum and the like.

[0064] The cultivation is generally conducted under 5% CO_2 at 37°C for 1 to 30 days. If necessary, an antibiotic such as kanamycin, penicillin or the like may be added to the medium during the cultivation.

[0065] After the cultivation, in the case where a microbial cell or a cell intracelluraly produced the IP_3 sponge of the invention, the IP_3 sponge is collected by disrupting the microbial cell or the cell by sonication, freezing and thawing method, or homogenizing. In the case where a microbial cell or a cell extracellularly produced the IP_3 sponge of the invention, the microbial cell or the cell is removed from the culture through centrifugation or the like before, or the culture solution is directly subjected to the isolation/purification procedure. The IP_3 sponge of the invention is isolated and purified from the culture through a general biochemical method for isolating and purifying a protein, such as ammonium sulfate precipitation, gel chromatography, ion exchange chromatography, affinity chromatography, or a combination thereof.

5. Therapeutic Agent and Agent for Gene Therapy

[0066] Since the protein and the gene of the invention has IP_3 neutralizing activity, they are useful as an antagonist for IP_3-induced calcium, a therapeutic agent and an agent for gene therapy for diseases associated with calcium production. The therapeutic agent or the agent for gene therapy of the invention can be administered orally or parenterally and systemically or locally.

[0067] When the protein or the gene of the invention is used as a therapeutic agent or an agent for gene therapy for disease associated with calcium production, the disease to be treated is not particularly limited. For example, the protein or the gene may be used for diseases in the nervous system, blood vascular system, respiratory system, digestive system, lymphatic system, urinary system, reproduction system or the like for the specific purpose of treatment or prevention. These diseases may be in the form of a single disease or may be complicated by one of these diseases or by some disease other than those mentioned above; any of such forms may be treated with the protein or the gene of the invention.

[0068] When the therapeutic agent of the invention is administered orally, the agent may be formulated into a tablet, capsule, granule, powder, pill, troche, internal liquid agent, suspension, emulsion, syrup or the like. Alternatively, the therapeutic agent may be prepared into a dry product which is re-dissolved just before use. When the therapeutic agent of the invention is administered parenterally, the agent may be formulated into a intravenous injection (including drops), intramuscular injection, intraperitoneal injection, subcutaneous injection, suppository, or the like. Injections are supplied in the form of unit dosage ampules or multi-dosage containers.

[0069] These formulations may be prepared by conventional methods using appropriate excipients, fillers, binders, wetting agents, disintegrating agents, lubricating agents, surfactants, dispersants, buffers, preservatives, dissolution aids, antiseptics, flavoring/perfuming agents, analgesics, stabilizers, isotonicity inducing agents, etc. conventionally used in pharmaceutical preparations.

[0070] Each of the above-described formulations may contain pharmaceutically acceptable carriers or additives. Specific examples of such carriers or additives include water, pharmaceutically acceptable organic solvents, collagen, polyvinyl alcohol, polyvinylpyrrolidone, carboxyvinyl polymers, sodium alginate, water-soluble dextran, sodium carboxymethyl amylose, pectin, xanthan gum, gum arabic, casein, gelatin, agar, glycerol, propylene glycol, polyethylene glycol, vaseline, paraffin, stearyl alcohol, stearic acid, human serum albumin, mannitol, sorbitol and lactose. One or a plurality of these additives are selected or combined appropriately depending of the form of the preparation.

[0071] The dosage levels of the therapeutic agent of the invention will vary depending on the age of the subject, the route of administration and the number of times of administration and may be varied in a wide range. When an effective amount of the protein of the invention is administered in combination with an appropriate diluent and a pharmaceutically acceptable carrier, the effective amount of the protein can be in the range from 0.0001 to 1000 mg/kg per administration. The therapeutic agent is administered once a day or in several dosages per day for at least one day.

[0072] When the gene of the invention is used as an agent for gene therapy for diseases associated with calcium production, the gene of the invention may be directly administered by injection. Alternatively, a vector incorporating the gene of the invention may be administered. Specific examples of a suitable vector for this purpose include an adenovirus vector, adeno-associated virus vector, herpes virus vector, vaccinia virus vector and retrovirus vector. The gene of the invention can be administered efficiently by using such a virus vector. Alternatively, the gene of the invention may be enclosed in phospholipid vesicles such as liposomes, and the resultant liposomes may be administered to the subject. Briefly, since liposomes are biodegradable material-containing closed vesicles, the gene of the invention is retained in the intemal aqueous layer and the lipid bilayer of liposomes by mixing the gene with the liposomes (a liposome-gene complex). Subsequently, when this complex is cultured with cells, the gene in the complex is taken into the cells (lipofection). Then, the resultant cells may be administered by the methods described below.

[0073] As a method for administering the agent for gene therapy of the invention, local administration to tissues of the central nervous system (brain, spiral cord), blood vascular system (artery, vein, heart), respiratory system (trachea, lung), digestive system (salivary glands, stomach, intestines, liver, pancreas), lymphatic system (lymph node, spleen, thymus), urinary system (kidney), reproduction system (testis, ovary, uterus) or the like may be performed in addition to conventional systemic administration such as intravenous or intra-arterial administration. Further, an administration method combined with catheter techniques and surgical operations may also be employed.

[0074] The dosage levels of the agent for gene therapy of the invention vary depending on the age, sex and conditions of the subject, the route of administration, the number of times of administration, and the type of the formulation. Usually, it is appropriate to administer the gene of the invention in an amount of 0.01-100 mg/adult body/day.

EXAMPLES

[0075] Hereinafter, the present invention will be described in detail by way of examples which do not limit the technical scope of the present invention.

Example 1: Construction of Expression Plasmid for High Affinity IP_3-Binding Polypeptide (IP_3 Sponge)

[0076] The N-terminal amino acids (734 amino acids) (polypeptide T734) of a mouse Type-1 IP_3 receptor (mIP_3R1) has a specific IP_3-binding activity. The cDNA portion coding for polypeptide T734 was cloned into *E.coli* expression vector pET-3a (whose expression is controlled by T7 promoter that is induced upon addition of IPTG) to obtain plasmid pET-T734 (Yoshikawa F. et al., *J. Biol. Chem.* 271:18277-18284, 1996). Using this plasmid (pET-T734) as a parent plasmid, the following expression plasmids were constructed for IP_3-binding polypeptides. Herein, an IP_3-binding polypeptide with high affinity is also referred to as an "IP_3 sponge".

(1-1) Expression plasmid for high affinity IP_3 sponge "T604"

[0077] A gene coding for polypeptide T604 that corresponds to the first methionine (M-1) to the lysine at Position 604 (K-604) of polypeptide T734 was prepared. Specifically, site-directed mutagenesis was conducted by PCR using a complementary oligonucleotide (Yoshikawa F. et al., *J Biol Chem,* 271:18277-18284, 1996) to introduce a stop codon (TAA) and a subsequent *Bam*HI recognition site (GGATCC) at Position 605 of T734.

Sense primer: 5'-TGTCAGA<u>CATATG</u>CGTGTTGGAA-3'

*Nde*I (SEQ ID NO: 5)

Antisense primer:

5'-CGCG<u>GGATCCTTA</u>TTTCCGGTTGTTGTGGAGCAGGG-3'

*Bam*HI (SEQ ID NO: 6)

[0078] The sense primer was introduced with a *Nde*I cleavage recognition sequence (CATATG) (underlined) including the first methionine codon (ATG).

[0079] A total of 100 µl PCR reaction solution was used. The PCR reaction solution contained 100 ng template DNA, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.2), 2 mM $MgCl_2$, 0.1% TritonX-100, 10 µg/ml BSA, 200 µM dNTPs, 1 µM sense primer, 1 µM anti-sense primer and 2.5 unit Pfu DNA polymerase. The PCR reaction was performed at 95°C for 1 min. and then through 30 cycles of: 95°C for 1 min.; 57°C for 3 min.; and 72°C for 3 min.

[0080] The 5'-end of the obtained amplified fragment was treated with *Nde*I and the 3'-end with *Bam*HI, thereby producing deletion mutant pET-T604 that contains DNA coding for an amino acid sequence corresponding to the amino acid sequence of T734 but with C-terminal deletion up to Position 605.

(1-2) Expression plasmid for high affinity IP_3 sponge "G224"

[0081] First, a gene coding for polypeptide T604 that corresponds to the first methionine (M-1) to the lysine at Position 604 (K-604) of polypeptide T734 was prepared. Specifically, site-directed mutagenesis was conducted by PCR using a complementary oligonucleotide (Yoshikawa F. et al., *J Biol Chem*, 271:18277-18284, 1996) to introduce a stop codon (TAA) and a subsequent *Eco*RI recognition site (GAATCC) at Position 605 of T734.

Sense primer: 5'-TGTCAGACATATGCGTGTTGGAA-3'

*Nde*I (SEQ ID NO: 5)

[0082] Antisense primer:

5'-CCGGAATTCTTATTTCCGGTTGTTGTGGAGCAGGG-3'

*Eco*RI (SEQ ID NO: 7)

[0083] The PCR was conducted under the same conditions as described in (1-1) above.

[0084] The 5'-end of the thus-obtained amplified fragment was treated with *Nde*I and the 3'-end with *Eco*RI, thereby producing deletion mutant pET-T604e that contains DNA coding for an amino acid sequence corresponding to the amino acid sequence of T734 but with C-terminal deletion up to Position 605.

[0085] Then, using deletion mutant pET-T604e as a template, site-directed mutagenesis was performed to introduce *Bam*HI recognition site (GGATCC) immediately before the methionine at Position 224 of polypeptide T604.

[0086] Antisense primer:

5'-CCGGAATTCTTATTTCCGGTTGTTGTGGAGCAGGG-3'

*Eco*RI (SEQ ID NO: 7)

[0087] Sense primer:

5'-CGCGGATCCATGAAATGGAGTGATAACAAAGACGACA-3'

*Bam*HI (SEQ ID NO: 8)

[0088] The PCR was conducted under the same conditions as described in (1-1) above.

[0089] The thus-obtained amplified fragment (plasmid introduced with mutation) was cleaved with *Bam*HI and *Eco*RI,

thereby obtaining a cDNA fragment coding for Amino acids 224-604. This cDNA fragment was ligated to *Bam*HI-*Eco*RI site of GST fusion plasmid (pGEX-2T) without a frameshift (in-frame), thereby obtaining plasmid pGEX-G224. Plasmid pGEX-G224 expresses fusion polypeptide G224 (Fig. 1) that includes polypeptide GST and subsequent polypeptide M-224 to K-604.

(1-3) Expression plasmid for low affinity IP_3-binding polypeptide

[0090] Site-directed mutagenesis was conducted by sequential PCR using pGEX-G224 as a template.
[0091] The following two mismatched oligonucleotides were synthesized to introduce mutation (K508A) at Position 508 of T604 where alanine was substituted for lysine (K-508):

5'-GAGAGCGGCAGGCACTGATGAGGG-3' (SEQ ID NO: 9)

5'-CCCTCATCAGTGCCTGCCGCTCTC-3' (SEQ ID NO: 10)

[0092] Using the above primers, site-directed mutagenesis was conducted by sequential PCR. The PCR conditions and the composition of the reaction solution were as follows:

Primary reaction 1

[0093]

Sense primer: 5'-GAGAGCGGCAGGCACTGATGAGGG-3' (SEQ ID NO: 9)

[0094] Antisense primer:

5'-CCGGAATTCTTATTTCCGGTTGTTGTGGAGCAGGG-3'
*Eco*RI (SEQ ID NO: 7)

[0095] The PCR was conducted under the same conditions as described in (1-1) above.

Primary reaction 2

[0096] Sense primer:

5'-CGCGGATCCATGAAATGGAGTGATAACAAAGACGACA-3'
*Bam*HI (SEQ ID NO: 8)

[0097] Antisense primer:

5'-CCCTCATCAGTGCCTGCCGCTCTC-3' (SEQ ID NO: 10)

[0098] The PCR was conducted under the same conditions as described in (1-1) above.

Secondary reaction

[0099] Ten µl of the PCR reaction product resulting through Primary reactions 1 and 2, and 1 µM each of primers (SEQ ID NOS: 7 and 8) were used to conduct PCR under the same conditions as the primary reactions.
[0100] The obtained amplified fragment was cleaved with *Bam*HI and *Eco*RI. The cleaved fragment was ligated to *Bam*HI-*Eco*RI site of GST fusion plasmid pGEX-2T without a frameshift (in frame), thereby obtaining plasmid pGEX-G224-m30. This mutant plasmid expresses polypeptide G224-m30 having the point mutation K508A (Fig. 1, m30).

(1-4) Expression plasmid for high affinity IP_3 sponge "G224-m49"

[0101] Using pGEX-G224 as a template, site-directed mutagenesis was conducted by sequential PCR.
[0102] The following two mismatched oligonucleotides were synthesized to introduce a mutation (R441Q) at Position 441 of T604 where glutamine was substituted for alginine (R-441):

5'-GCTGAGGTTCAAGACCTGGACTTTG-3' (SEQ ID NO: 11)

5'-AAAGTCCAGGTCTTGAACCTCAGC-3' (SEQ ID NO: 12)

Primary reaction 1

[0103] Sense primer:

5'-GCTGAGGTTCAAGACCTGGACTTTG-3' (SEQ ID NO: 11)

[0104] Antisense primer:

5'-CCGGAATTCTTATTTCCGGTTGTTGTGGAGCAGGG-3'
*Eco*RI (SEQ ID NO: 7)

[0105] The PCR was conducted under the same conditions as described in (1-3) above.

Primary reaction 2

[0106] Sense primer:

5'-CGCGGATCCATGAAATGGAGTGATAACAAAGACGACA-3'

*Bam*HI (SEQ ID NO: 8)

[0107] Antisense primer:

5'-AAAGTCCAGGTCTTGAACCTCAGC-3' (SEQ ID NO: 12)

[0108] The PCR was conducted under the same conditions as described in (1-3) above.

Secondary reaction

[0109] Ten μl of the PCR reaction product resulting through Primary reactions 1 and 2, and 1 μM each of primers (SEQ ID NOS: 6 and 8) were used to conduct PCR under the same conditions as those of the primary reactions.
[0110] The obtained amplified fragment was cleaved at a *Bam*HI-*Eco*RI site. The cleaved fragment was ligated to *Bam*HI-*Eco*RI site of GST fusion plasmid pGEX-2T without a frameshift (in frame), thereby obtaining plasmid pGEX-G224-m49. This mutant plasmid expresses polypeptide G224-m49 having the point mutation R441Q (Fig. 1, m49).

Example 2: Expression and Preparation of

High Affinity IP_3-Binding Polypeptide with *E.coli*

[0111] Since the IP_3-binding core mostly results in insoluble inclusion bodies, the expression amount is low. Thus, the present inventors have modified the IP_3-binding region through gene engineering to produce a high affinity IP_3-binding polypeptide which is of lower molecule, which is capable of stable mass-expression, which can be recovered as a soluble protein, which has a higher affinity, and which has as high specificity as a conventional IP_3 receptor.
[0112] By low-temperature cultivation (16-22°C), polypeptide T734 can be mass-expressed in a stable manner with a relatively high soluble fraction recovery (Kd = 50 ± 2.4 nM, B_{max} = 46 pmol/mg protein, 1.85 mg/l *E.coli* culture (corresponding to about 0.5 g of wet *E.coli*)). However, the inclusion bodies amount to more than ten times the amount of the soluble fraction (Yoshikawa F. et al., *J. Biol Chem.* 271: 18277-18284, 1996).
[0113] First of all, smaller polypeptides that had the above-described characteristics were prepared.
[0114] The pET-type and pGEX-type expression plasmids obtained in Example 1 were introduced into *E.coli* BL21 (DE3) and JM109, respectively, by transformation method. Expression induction with IPTG and preparation of expression proteins from *E.coli* were mainly conducted by modifying the method of Yoshikawa et al (Yoshikawa F. et al., *J. Biol Chem.* 271: 18277-18284, 1996).
[0115] Specifically, *E.coli* introduced with respective plasmids were shake cultured in L broths (containing 100 μg/ml ampicillin) at 22°C. When the absorption OD_{600} became about 1.5, IPTG was added to 0.5 mM. After a few hours of shake culture at 16°C, each of the *E.coli* was recovered through centrifugation and suspended in PBS containing protease inhibitors (1 mM PMSF, 10 μM leupeptin, 1 μM pepstatin A, 2 μg/ml aprotinin). Each of the *E.coli* was disrupted by sonication. Then, each supernatant containing the expression polypeptide (soluble fraction) was collected by ultracentrifugation (Beckman Ti35 rotor, 25,000 rpm, 1 hr., 4°C).
[0116] GST fusion polypeptides were purified from the soluble fractions by affinity purification using Glutathione-Sepharose column (Pharmacia LKB). Specifically, each of the GST fusion polypeptides was eluted from the column with 10 mM glutathione/50 mM Tris-HCl (pH 8.0) by mainly following the manual provided by the manufacturer. The polypeptide solutions were equilibrated with 10 mM HEPES-KOH (pH 7.2), 88 mM NaCl and 1 mM KCl using PD10 desalted column (Pharmacia LKB), and then dispensed, thereby obtaining the IP_3 sponges (Fig. 1: G224, m30, m49 and GST). The IP_3 sponges were stored at -80°C until they were used.
[0117] A series of deletion mutants based on polypeptide T734 were prepared by serially shortening the length of the polypeptide T734 from the C-terminus. The analysis of the deletion mutants indicated that T705 and T699 had no marked characteristic difference with T734. In the cases of polypeptides T569, T572 and T576, the expression amounts of the soluble proteins were lower than T734. Stable mass-expression of soluble protein was successful with polypeptide T604 which was obtained by deleting the C-terminus of T734 up to Amino acid 605 (Fig. 1).
[0118] With reference to Fig. 1, the uppermost (IP_3R1) is the N-terminal amino acids of the IP_3 receptor including the IP_3-binding core region (core: Amino acids 226-578). T604 (Amino acids 1-604), G224 (GST + Amino acids 224-604), G224m30 (G224 introduced with K508A mutation), G224m49 (G224 introduced with R441Q mutation), and GST (derived from pGEX-2T)) are also shown in Fig. 1.
[0119] T604 had a $[^3H]IP_3$-binding activity substantially equivalent to that of T734 (Kd = 45 nM), and a higher yield of soluble protein (B_{max} = 690 pmol/mg protein). Specifically, the yield was 19 mg/l *E.coli* culture (Figs. 2B and 2C, Table 1).

Table 1

Expression of IP_3-binding site in *E.coli*			
Protein	Expression efficiency (mg/l *E.coli* culture)	Kd [nM]	B_{max} [pmol/μg purified protein]
Purified IP_3R[a]	-	83	2.1

a. Maeda et al., *EMBO J.* 9, 51-67, 1990

Table 1 (continued)

Expression of IP_3-binding site in *E.coli*			
Protein	Expression efficiency (mg/l *E.coli* culture)	Kd [nM]	B_{max} [pmol/μg purified protein]
T734[b]	1.85	(50)[c]	ND
T604	19	7.6/(45)c	ND
G224	30	0.083	1.6
G224m49	ND	0.043	1.7
G224m30	ND	330	3.0

b. Yoshikawa et al., *J. Biol. Chem.*, 271, 18277-18284, 1996

c. the values in parentheses represent Kd obtained from crude cell lysates

ND. Not Determined

[0120] The total expression amount of polypeptide T604 substantially equaled to that of polypeptide T734 but T604 had a remarkably improved soluble protein yield. The yield of soluble protein of polypeptide T604 was substantially the same at 30°C and 37°C, and reached the peak within 2 hours after initiating expression induction.

[0121] Fig. 2A shows the result of Western blotting analysis of the protein (0.1 μg) obtained from an *E.coli* extract solution (soluble fraction) that expresses polypeptide T604 (66kDa). As a control, a cell extract solution obtained by transforming a vector that does not include T604 (pET-3a) was used. Fig. 2B shows a comparison of the total amounts of specific IP_3-binding contained in 0.7 μl soluble fractions, for T734, T604, and the control vector. Fig. 2C shows the result of Schatchard plot analysis where the binding between 3 μg of T604 soluble fraction and 9.6 nM $[^3H]IP_3$ was competitively inhibited with non-labeled IP_3 (cold IP_3) at various concentrations. The results were Kd = 45 ± 7.6, B_{max} = 690 ± 64 pmol/mg protein.

[0122] When T734 was serially deleted from the N-terminus, a very short N-terminal deletion of T734 (e.g., a deletion of 31 amino acids) caused lack of IP_3-binding activity even the deletion was outside the core region. However, the polypeptide retrieved the IP_3-binding activity when the N-terminus was deleted to Amino acid 220-225, near the N-terminus of the core region (Yoshikawa et al, 1996). The theory for this is unknown, but presumably, the formation of the three-dimensional structure for active core region is somehow interrupted depending on the degree of deletion. Although the active polypeptide with the N-terminal deletion up to Amino acid 220-225 had a relatively high affinity, the amount of soluble protein expressed was lower.

[0123] As described above, a protein obtained by deleting Amino acids 1-223 of polypeptide T604 (N4-T604; Amino acids 224-604) had a higher activity (about 3 times high) but lower production than those of the original T604. Accordingly, polypeptide T604 seemed to be the most suitable polypeptide for stably mass-expressing only the high affinity IP_3-binding region as a soluble protein.

Example 3: Expression of IP_3 Sponge

(i) $[^3H]$-IP_3-binding inhibition experiment

[0124] Based on the results obtained in Example 2, an IP_3-binding polypeptide with a higher affinity was produced. As described above, when the amino-terminal Amino acids 1-223 of polypeptides T604 and T734 were deleted, high $[^3H]IP_3$-binding activities were obtained. Even Amino acid region 224-579 (a polypeptide that almost corresponds to the core region) consisting of only 356 amino acid residues has an affinity as high as Kd = 2.3 nM (Yoshikawa et al., 1996, *supra*). However, as described above, these polypeptides have lower soluble protein expression levels. In other words, longer amino terminal deletion may result in a higher affinity on one hand, but it also lowers the expression amount and expression stability of soluble proteins by rendering most of proteins as insoluble inclusion bodies.

[0125] In general, stability, solubility and an expression level of a foreign polypeptide are known to be improved when it is made into a GST fusion body. In this example, fusion proteins G224, G224-m30 and G224-m49 consisting of GST and an IP_3-binding site (Amino acid region 224-604) were prepared by ligating GST to replace the N-terminal region (Amino acids 1-223) of the IP_3 receptor (Fig. 1).

[0126] The IP_3-binding activities of these fusion proteins were measured mainly by the method of Yoshikawa et al (1996).

[0127] Each fusion protein (IP_3 sponge) (0.2 μg) was mixed with 100 μl of binding buffer-α (50 mM Tris-HCl (pH 8.0 at 4°C), 1 mM EDTA, 1 mM β-mercaptoethanol) that contained 9.6 nM D-myo-$[^3H](1,4,5)IP_3$ (777 GBq/mmol; DuPont NEN) (hereinafter, abbreviated as "$[^3H]IP_3$") and various concentrations of non-labeled D-myo-$(1,4,5)IP_3$ (Dojindo) (hereinafter, abbreviated as "cold IP_3"). The mixture was left to stand on ice for 10 minutes. To the mixture, 4 μl of 50

mg/ml γ-globulin (Sigma) (final concentration: 1 mg/ml) and 100 μl of 30% PEG 6000 (Sigma)/binding buffer-α solution (final concentration: 15%) were added. The resultant mixture was left to stand on ice for 5 minutes, and then centrifuged at 10,000 x g at 2°C for 5 minutes to collect polypeptide/PEG complex. PEG-precipitated [^{3}H]IP$_3$-binding polypeptide was well solubilized with 180 μl solubilizer Solvable (DuPont NEN). The resultant was neutralized with 18 μl glacial acetic acid and then added to 5 ml liquid scintillation counter (Atomlight [DuPont NEN]) to measure the radioactivity (first radioactivity). Nonspecific binding of each protein was determined by measuring the second radioactivity in the presence of 2 μM or 10 μM cold IP$_3$. Then, a specific binding value of each protein was obtained by subtracting the second radioactivity (nonspecific binding value) from the first radioactivity values.

[0128] Scatchard plot analysis was conducted under the following conditions. For low-affinity polypeptides (G224-m30 and control GST), the binding experiment was conducted in 100 μl binding buffer α by adding 9.6 nM [^{3}H]IP$_3$ (DuPont NEN) and 10-20 nM of cold IP$_3$ to 2 μg of IP$_3$-binding polypeptide, and by adding 9.6 nM [^{3}H]IP$_3$ (DuPont NEN) and 50 nM-2 μM of cold IP$_3$ to 0.01 μg IP$_3$-binding polypeptide. For high-affinity IP$_3$ sponges (G224 and G224-m49), binding experiment was conducted with 0.02 μg IP$_3$ sponges at [^{3}H]-IP$_3$ concentrations of 0.15, 0.3, 0.6, 1.2, 2.4, 4.8 and 9.6 nM without adding cold IP$_3$.

[0129] The inhibition effects of the IP$_3$-binding polypeptides (IP$_3$ sponges) on [^{3}H]IP$_3$-binding activity of cerebellar microsome was analyzed as follows.

[0130] A microsomal fraction was prepared from the cerebella of mice *ddY* (Nippon SLC) mainly by following the method of Nakada et al. (Nakada S. et al., *Biochem. J.* 277:125-131, 1991). In 100 μl of binding buffer α, various concentrations of the IP$_3$ sponges were added respectively to see the changes in the binding between the cerebellar microsome (40 μg) and 9.6 nM [^{3}H]-IP$_3$ according to the above method (see Scatchard plot analysis).

[0131] As a result, the affinity of polypeptide G224 was found out to be 500 times higher than that of polypeptide T734 (Kd = 83 pM, B_{max} = 1.6 pmol/μg protein) (Fig. 3A). Polypeptide G224 binds well to (1,4,5)IP$_3$ and (2,4,5)IP$_3$ and the yield of IP$_3$-binding protein was about 30 mg/l *E.coli* culture (Table 1). After purifying the protein with a glutathione column and a subsequent PD10 column, the yield was about 24 mg/l. The binding activity was augmented when R441Q mutation was introduced into polypeptide T734 (Yoshikawa et al., 1996, *supra*). The affinity of polypeptide G224-m49 (G224 introduced with R441Q mutation) doubled and became about 1,000 times higher than that of polypeptide T734 (Kd = about 43 pM, B_{max} = 1.7 pmol/μg protein) (Fig. 3B, Table 1). The binding activity decreased when polypeptide T734 was introduced with K508A mutation (Yoshikawa et al., 1996 (*supra*)). Similarly, the binding activity of polypeptide G224-m30 decreased when G224 was introduced with K508A mutation and became as low as about 1/4,000 of polypeptide G224 and about 1/7,700 of polypeptide G224-m49 (Kd = about 330 nM, B_{max} = 3.0 pmol/μg protein) (Fig. 3C, Table 1).

(ii) IP$_3$-binding inhibition via absorption by novel IP$_3$ sponge

[0132] IP$_3$-binding polypeptides G224 and G224-m49 have powerful IP$_3$-binding activities that are 500 to 1,000 times higher than that of the original IP$_3$ receptor. Polypeptides G224 and G224-m49 were tested for their use as an IP$_3$-specific absorption body (sponge) (IP$_3$ sponge), i.e., whether they can decrease the amount of IP$_3$-binding by the IP$_3$ receptors in a solution by competitively absorbing IP$_3$ in the solution (Fig. 4).

[0133] Mouse cerebellum is a tissue that is rich in IP$_3$ receptor and whose microsomal fraction has a [^{3}H]IP$_3$-binding activity which is at least 50 times higher than those in other tissues (Maeda et al., 1990 (*supra*)). Binding between 40 μg cerebellar microsome (Kd = 21 nM, B_{max} = 23 pmol/mg protein) and 9.6 nM [^{3}H]IP$_3$ in 100 μl solution was analyzed for percentage (%) of competitive inhibition at various concentrations of IP$_3$ sponges where the activity under the absence of IP$_3$ sponge was considered 100%. It was calculated that, there were about 0.92 pmol of IP$_3$-binding site of cerebellum IP$_3$ receptor and 0.96 pmol of [^{3}H]IP$_3$ present in the 100 μl solution.

[0134] As a result, no inhibition effect was observed for control GST even when the IP$_3$ sponge concentration was 100 pg/ml (Fig. 4). On the other hand, for high-affinity polypeptides G224 and G224-m49, strong IP$_3$-binding inhibition activities were observed and IC_{50} was about 10 μg/ml (Fig. 4). Polypeptide G224-m30 with low affinity had low inhibition activity with IC_{50} of 100 μg/ml. According to this *in vitro* experiment system, the IP$_3$ sponge tended to precipitate with the microsome membrane when the IP$_3$ sponge concentration exceeded about 25 μg/ml, and so the concentration-dependent curves were likely to fluctuate (Fig. 4). Thus, the apparent inhibition of G224-m30 observed at IP$_3$ sponge concentration exceeding 25 μg/ml could be due to precipitation under high concentration.

[0135] These results show that [^{3}H]IP$_3$-binding of the IP$_3$ receptor can efficiently be inhibited according to the binding affinity and the concentration of the IP$_3$ sponge used. High affinity IP$_3$-binding polypeptide of the invention is a novel IP$_3$ sponge that can be used as an IP$_3$ neutralizing agent, or an antagonist for IP$_3$-induced calcium.

Example 4: Test of Inhibiting IP$_3$-Induced Ca^{2+} Release (IICR)

[0136] To conduct a test of inhibiting IP$_3$-induced Ca^{2+} release, a microsomal fraction was prepared from mouse

cerebellum as described in Example 3. The fraction was suspended in Buffer B, dispensed, and stored at -80°C until it was used.

[0137] Composition of Buffer B was 110 mM KCl, 10 mM NaCl, 5 mM KH_2PO_4, 1 mM DDT, and 50 mM HEPES-KOH (pH 7.2) (containing a cocktail of protease inhibitors [0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A, 10 μM E-64] and 2 mM $MgCl_2$).

[0138] An IP_3-induced Ca^{2+} release activity of cerebellar microsome was determined by using fura-2 (Molecular Probe) as a fluorescent Ca^{2+} indicator. Specifically, excitations upon addition of IP_3 at two wavelengths (340 nm and 380 nm) were measured with fluorescence spectrophotometer CAF110 (Nihon Bunko) to see the change in the fluorescent intensity ratio (F340/F380) at 500 nm.

[0139] IP_3-induced Ca^{2+} release from the cerebellar microsome is generally EC_{50} = 100-200 nM IP_3. Cerebellar microsome (100 μg) was mixed with 500 μl of a release buffer (Buffer B containing 1 mM $MgCl_2$, 2 μM fura-2, 1 mM DTT, 10 mM creatine phosphate, 40U/ml creatine kinase, 1 μg/ml oligomycin, and the cocktail of protease inhibitors) in a measurement cuvette with a stirrer bar. The following reaction was conducted at 30°C while constantly stirring with the stirrer bar.

[0140] One mM of ATP was added to the mixture in the cuvette to activate Ca^{2+} pumping (Ca^{2+}-ATPase), whereby Ca^{2+} was incorporated into the inner space of microsome (Ca^{2+} loading). Ca^{2+} loading was confirmed by monitoring until the decrease of fura-2 fluorescent level became constant. The change in the fura-2 fluorescent intensity ratio was measured (F340/F380) at a subthreshold level.

[0141] The effect of IP_3 sponge on inhibiting IP_3-induced Ca^{2+} release activity of cerebellar microsome was analyzed as follows. After the addition of ATP, the curve of fura-2 fluorescent intensity was monitored until the decrease became constant. Then, various concentrations of IP_3 sponges were added. After 1 min., 50 nM to 1 μM of IP_3 was added to the reaction mixture to observe the change of fura-2 fluorescent intensity induced by the IP_3.

[0142] The IP_3 sponge concentration dependency was determined as follows. High affinity polypeptide G224 of 3.125, 6.25, 12.5, 25, 50, 100, 200 μg/ml were added to the reaction mixture, respectively. After about 1 min., 100 nM of IP_3 was added to measure the Ca^{2+} release activity induced by the IP_3. The concentration dependency of low affinity polypeptide G224-m30 was determined by adding G224-m30 of 200, 400 and 500 μg/ml. After about 1 min., 100 nM of IP_3 was added to measure the Ca^{2+} release activity induced by the IP_3. In addition, G224-m30 of 500 μg/ml was also added, and after about 1 min., 50 nM of IP_3 was added to measure the Ca^{2+} release activity induced by the IP_3.

[0143] As a result, it was found that the IP_3 sponges specifically inhibited in a competitive manner the IP_3-binding by the IP_3 receptor of cerebellar microsome by absorbing the IP_3 (Figs. 5A-5F, 6A-6G and 7). In Figs. 5A-5F and 6A-6G, the vertical axis represent the change in fura-2 fluorescent intensity ratio (F340/F380) (i.e., change in the amount of Ca^{2+}), and the horizontal axis represents the time (sec).

[0144] As shown in Figs. 5A, 5B and 5C, in the absence of IP_3 sponge (controls), IP_3-induced Ca^{2+} release activities were dependent on IP_3 concentration. Low-affinity polypeptide G224-m30 at a concentration of 500 μg/ml had no inhibiting effect on Ca^{2+} release with 100 nM IP_3 (Fig. 5E). Little difference was found between G224-m30 and the control for effects on inhibiting 50 nM IP_3 (Fig. 5E). With GST only, even at a high concentration of 632 μg/ml, no change was seen in Ca^{2+} release activity induced with 50 nM IP_3 (Fig. 5F). Thus, in each case, no marked difference was noted with the control.

[0145] On the contrary to the above results, high affinity polypeptide G224 had a significant inhibition effect on IP_3-induced Ca^{2+} release depending on its concentration (Figs. 6A-6G). The high affinity polypeptide G224 had the greatest inhibition effect at 100 μg/ml and almost completely inhibited the IP_3-induced Ca^{2+} release (Fig. 6F).

[0146] The peak values of Ca^{2+} release obtained by adding G224 at each concentration shown in Figs. 6A-6G, were plotted where the peak obtained in the absence of G224 was considered 100 % (Fig. 7). The horizontal axis represents each concentration of polypeptide G224 and the verticle axis represents the peak value of Ca^{2+} release. As can be appreciated from Fig. 7, the concentration of polypeptide G224 required for 50 % inhibition of IP_3-induced Ca^{2+} release was about 20 μg/ml.

[0147] Accordingly, it was found that the high affinity IP_3-binding polypeptide acted as an IP_3 sponge and specifically inhibited, in a concentration-dependant manner, the IP_3-induced Ca^{2+} release by the IP_3 receptor on cerebellar microsome.

[0148] The present invention provides a polypeptide having a high affinity binding activity to inositol 1,4,5-trisphosphate, a gene encoding the polypeptide, a recombinant vector including the gene, a transformant including the vector and a method for producing the polypeptide.

[0149] The polypeptide of the invention can be used to control the inhibition of a specific cell function that depends on an IP_3-induced calcium signal transmission (IP_3 neutralizing agent, antagonist for IP_3-induced calcium, etc.). Furthermore, the polypeptide and the gene of the present invention is useful as an IP_3 signal detecting agent for inhibiting activation of IP_3-induced calcium signal transmission. The gene of the invention is also useful as a therapeutic agent for treating a disease associated with calcium production.

[0150] Various other modifications will be apparent to and can be readily made by those skilled in the art without

departing from the scope and spirit of this invention. Accordingly, it is not intended that the scope of the claims appended hereto be limited to the description as set forth herein, but rather that the claims be broadly construed.

[0151] All publications, patents, and patent applications cited herein are incorporated herein by reference in their entirety.

[0152] The following are information on sequences described herein:

Annex to the description

[0153]

SEQUENCE LISTING

<110> RIKEN ; Katsuhiko Mikoshiba

<120> High affinity IP3 binding polypeptide

<130> PH-657EP

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<150> JP98/242207

<151> 1998-08-27

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<170> PatentIn Ver. 2.0

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ctg tat gcg gag gga tct acg aat gga ttt atc agc acc tta ggc ttg    96
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             20                  25                  30

gtt gat gac cgt tgt gtt gta cag cca gaa gcc ggg gac ctt aac aat   144
Val Asp Asp Arg Cys Val Val Gln Pro Glu Ala Gly Asp Leu Asn Asn
         35                  40                  45

cca ccc aag aaa ttc aga gac tgc ctc ttt aag cta tgt cct atg aat   192
Pro Pro Lys Lys Phe Arg Asp Cys Leu Phe Lys Leu Cys Pro Met Asn
     50                  55                  60
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cga tac tcc gca cag aaa cag ttc tgg aaa gct gct aag ccc ggg gcc 240
Arg Tyr Ser Ala Gln Lys Gln Phe Trp Lys Ala Ala Lys Pro Gly Ala
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aac agc act aca gat gca gtg ctg ctc aac aaa ttg cat cat gct gca 288
Asn Ser Thr Thr Asp Ala Val Leu Leu Asn Lys Leu His His Ala Ala
85 90 95

gac ttg gaa aag aag cag aat gag aca gaa aac agg aaa ttg ttg ggg 336
Asp Leu Glu Lys Lys Gln Asn Glu Thr Glu Asn Arg Lys Leu Leu Gly
100 105 110

acc gtc atc caa tat ggc aac gtg atc cag ctc ctg cat ttg aaa agc 384
Thr Val Ile Gln Tyr Gly Asn Val Ile Gln Leu Leu His Leu Lys Ser
115 120 125

aat aaa tac ctg act gtg aat aag agg ctc cca gcc ttg cta gag aag 432
Asn Lys Tyr Leu Thr Val Asn Lys Arg Leu Pro Ala Leu Leu Glu Lys
130 135 140

aat gcc atg agg gtg acg ttg gac gag gct gga aat gaa ggg tcc tgg 480
Asn Ala Met Arg Val Thr Leu Asp Glu Ala Gly Asn Glu Gly Ser Trp
145 150 155 160

ttt tac att caa cca ttt tac aag ctt cgc tcc atc gga gac agt gtg 528
Phe Tyr Ile Gln Pro Phe Tyr Lys Leu Arg Ser Ile Gly Asp Ser Val
165 170 175

gtc ata ggc gac aag gta gtt ttg aat cct gtc aat gct ggc cag cct 576
Val Ile Gly Asp Lys Val Val Leu Asn Pro Val Asn Ala Gly Gln Pro
180 185 190

cta cat gcc agc agt cat cag ctg gtg gat aac cca ggc tgc aat gag 624
Leu His Ala Ser Ser His Gln Leu Val Asp Asn Pro Gly Cys Asn Glu
195 200 205

gtc aac tcc gtc aac tgt aat aca agc tgg aag ata gtg ctt ttc atg 672
Val Asn Ser Val Asn Cys Asn Thr Ser Trp Lys Ile Val Leu Phe Met

210 215 220

aaa tgg agt gat aac aaa gac gac att ctc aaa gga ggt gat gtg gtg 720

Lys Trp Ser Asp Asn Lys Asp Asp Ile Leu Lys Gly Gly Asp Val Val

225 230 235 240

agg ctc ttc cat gcc gag caa gag aag ttt ctc acc tgt gat gag cac 768

Arg Leu Phe His Ala Glu Gln Glu Lys Phe Leu Thr Cys Asp Glu His

245 250 255

cgg aag aag cag cat gtg ttc ctg agg acc acc ggc agg cag tca gcc 816

Arg Lys Lys Gln His Val Phe Leu Arg Thr Thr Gly Arg Gln Ser Ala

260 265 270

acg tcg gcc acc agt tct aaa gcc ctg tgg gaa gtg gag gta gtc cag 864

Thr Ser Ala Thr Ser Ser Lys Ala Leu Trp Glu Val Glu Val Val Gln

275 280 285

cac gac cca tgt cgg ggt gga gct ggg tac tgg aat agc ctc ttc cgg 912

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Phe Lys His Leu Ala Thr Gly His Tyr Leu Ala Ala Glu Val Asp Pro

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Asp Phe Glu Glu Glu Cys Leu Glu Phe Gln Pro Ser Val Asp Pro Asp

325 330 335

cag gat gca tct cgg agt agg ttg aga aac gcg caa gaa aaa atg gta 1056

Gln Asp Ala Ser Arg Ser Arg Leu Arg Asn Ala Gln Glu Lys Met Val

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Tyr Ser Leu Val Ser Val Pro Glu Gly Asn Asp Ile Ser Ser Ile Phe

355 360 365

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Glu Leu Asp Pro Thr Thr Leu Arg Gly Gly Asp Ser Leu Val Pro Arg
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Asn Ser Tyr Val Arg Leu Arg His Leu Cys Thr Asn Thr Trp Val His
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Ser Thr Asn Ile Pro Ile Asp Lys Glu Glu Glu Lys Pro Val Met Leu
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Lys Ile Gly Thr Ser Pro Leu Lys Glu Asp Lys Glu Ala Phe Ala Ile
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Val Pro Val Ser Pro Ala Glu Val Arg Asp Leu Asp Phe Ala Asn Asp
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gcc agc aag gtg ctg ggc tcc atc gct ggg aag ttg gaa aag ggc acc 1392
Ala Ser Lys Val Leu Gly Ser Ile Ala Gly Lys Leu Glu Lys Gly Thr
450 455 460

atc acc cag aat gag aga agg tct gtc acg aag ctt ttg gaa gac ttg 1440
Ile Thr Gln Asn Glu Arg Arg Ser Val Thr Lys Leu Leu Glu Asp Leu
465 470 475 480

gtt tac ttt gtc acg ggt gga act aac tct ggc caa gac gtg ctt gaa 1488
Val Tyr Phe Val Thr Gly Gly Thr Asn Ser Gly Gln Asp Val Leu Glu
485 490 495

gtt gtc ttc tct aag ccc aat cga gag cgg cag aag ctg atg agg gaa 1536
Val Val Phe Ser Lys Pro Asn Arg Glu Arg Gln Lys Leu Met Arg Glu
500 505 510

cag aat att ctc aag cag atc ttc aag ctg ttg cag gcc ccc ttc acg 1584
Gln Asn Ile Leu Lys Gln Ile Phe Lys Leu Leu Gln Ala Pro Phe Thr
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gac tgc ggg gat ggc ccg atg ctt cgg ctg gag gag ctg ggg gat cag 1632

Asp Cys Gly Asp Gly Pro Met Leu Arg Leu Glu Glu Leu Gly Asp Gln

530 535 540

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Arg His Ala Pro Phe Arg His Ile Cys Arg Leu Cys Tyr Arg Val Leu

545 550 555 560

cga cac tca cag caa gac tac agg aag aac cag gag tac ata gcc aag 1728

Arg His Ser Gln Gln Asp Tyr Arg Lys Asn Gln Glu Tyr Ile Ala Lys

565 570 575

cag ttt ggc ttc atg cag aag cag att ggc tat gac gtg ctg gcc gaa 1776

Gln Phe Gly Phe Met Gln Lys Gln Ile Gly Tyr Asp Val Leu Ala Glu

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35 40 45

Pro Pro Lys Lys Phe Arg Asp Cys Leu Phe Lys Leu Cys Pro Met Asn

50 55 60

Arg Tyr Ser Ala Gln Lys Gln Phe Trp Lys Ala Ala Lys Pro Gly Ala

65 70 75 80

Asn Ser Thr Thr Asp Ala Val Leu Leu Asn Lys Leu His His Ala Ala

85 90 95

Asp Leu Glu Lys Lys Gln Asn Glu Thr Glu Asn Arg Lys Leu Leu Gly

100 105 110

Thr Val Ile Gln Tyr Gly Asn Val Ile Gln Leu Leu His Leu Lys Ser

115 120 125

Asn Lys Tyr Leu Thr Val Asn Lys Arg Leu Pro Ala Leu Leu Glu Lys

130 135 140

Asn Ala Met Arg Val Thr Leu Asp Glu Ala Gly Asn Glu Gly Ser Trp

145 150 155 160

Phe Tyr Ile Gln Pro Phe Tyr Lys Leu Arg Ser Ile Gly Asp Ser Val

165 170 175

Val Ile Gly Asp Lys Val Val Leu Asn Pro Val Asn Ala Gly Gln Pro

180 185 190

Leu His Ala Ser Ser His Gln Leu Val Asp Asn Pro Gly Cys Asn Glu

195 200 205

Val Asn Ser Val Asn Cys Asn Thr Ser Trp Lys Ile Val Leu Phe Met

210 215 220

Lys Trp Ser Asp Asn Lys Asp Asp Ile Leu Lys Gly Gly Asp Val Val

225 230 235 240

Arg Leu Phe His Ala Glu Gln Glu Lys Phe Leu Thr Cys Asp Glu His

245 250 255

Arg Lys Lys Gln His Val Phe Leu Arg Thr Thr Gly Arg Gln Ser Ala

260 265 270

Thr Ser Ala Thr Ser Ser Lys Ala Leu Trp Glu Val Glu Val Val Gln

275 280 285

His Asp Pro Cys Arg Gly Gly Ala Gly Tyr Trp Asn Ser Leu Phe Arg

290 295 300

Phe Lys His Leu Ala Thr Gly His Tyr Leu Ala Ala Glu Val Asp Pro

305 310 315 320

Asp Phe Glu Glu Glu Cys Leu Glu Phe Gln Pro Ser Val Asp Pro Asp

325 330 335

Gln Asp Ala Ser Arg Ser Arg Leu Arg Asn Ala Gln Glu Lys Met Val

340 345 350

Tyr Ser Leu Val Ser Val Pro Glu Gly Asn Asp Ile Ser Ser Ile Phe

355 360 365

Glu Leu Asp Pro Thr Thr Leu Arg Gly Gly Asp Ser Leu Val Pro Arg

370 375 380

Asn Ser Tyr Val Arg Leu Arg His Leu Cys Thr Asn Thr Trp Val His

385 390 395 400

Ser Thr Asn Ile Pro Ile Asp Lys Glu Glu Glu Lys Pro Val Met Leu

405 410 415

Lys Ile Gly Thr Ser Pro Leu Lys Glu Asp Lys Glu Ala Phe Ala Ile

420 425 430

Val Pro Val Ser Pro Ala Glu Val Arg Asp Leu Asp Phe Ala Asn Asp

435 440 445

Ala Ser Lys Val Leu Gly Ser Ile Ala Gly Lys Leu Glu Lys Gly Thr

450 455 460

Ile Thr Gln Asn Glu Arg Arg Ser Val Thr Lys Leu Leu Glu Asp Leu

465 470 475 480

Val Tyr Phe Val Thr Gly Gly Thr Asn Ser Gly Gln Asp Val Leu Glu

485 490 495

Val Val Phe Ser Lys Pro Asn Arg Glu Arg Gln Lys Leu Met Arg Glu

500 505 510

Gln Asn Ile Leu Lys Gln Ile Phe Lys Leu Leu Gln Ala Pro Phe Thr

515 520 525

Asp Cys Gly Asp Gly Pro Met Leu Arg Leu Glu Glu Leu Gly Asp Gln

530 535 540

Arg His Ala Pro Phe Arg His Ile Cys Arg Leu Cys Tyr Arg Val Leu

545 550 555 560

Arg His Ser Gln Gln Asp Tyr Arg Lys Asn Gln Glu Tyr Ile Ala Lys

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Gln Phe Gly Phe Met Gln Lys Gln Ile Gly Tyr Asp Val Leu Ala Glu

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ccgctcccat cctaacggaa cgagctccct cttcgcggac atgggattgc ccagcggctg 180

ctaacccctc tcctggtcct gatcccccaa accggcgtgg ctccccggtc accaaggagc 240

tgattacaag ggaccaggat ttgcatcctt ggctgggcgt ccattggcta cagagtgcct 300

gacctgggtc aggctttcca acacggac atg tct gac aaa atg tcg agt ttc 352

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Leu His Ile Gly Asp Ile Cys Ser Leu Tyr Ala Glu Gly Ser Thr Asn
     10                  15                  20
gga ttt atc agc acc tta ggc ttg gtt gat gac cgt tgt gtt gta cag    448
Gly Phe Ile Ser Thr Leu Gly Leu Val Asp Asp Arg Cys Val Val Gln
 25                  30                  35                  40
cca gaa gcc ggg gac ctt aac aat cca ccc aag aaa ttc aga gac tgc    496
Pro Glu Ala Gly Asp Leu Asn Asn Pro Pro Lys Lys Phe Arg Asp Cys
                 45                  50                  55
ctc ttt aag cta tgt cct atg aat cga tac tcc gca cag aaa cag ttc    544
Leu Phe Lys Leu Cys Pro Met Asn Arg Tyr Ser Ala Gln Lys Gln Phe
             60                  65                  70
tgg aaa gct gct aag ccc ggg gcc aac agc act aca gat gca gtg ctg    592
Trp Lys Ala Ala Lys Pro Gly Ala Asn Ser Thr Thr Asp Ala Val Leu
         75                  80                  85
ctc aac aaa ttg cat cat gct gca gac ttg gaa aag aag cag aat gag    640
Leu Asn Lys Leu His His Ala Ala Asp Leu Glu Lys Lys Gln Asn Glu
     90                  95                  100
aca gaa aac agg aaa ttg ttg ggg acc gtc atc caa tat ggc aac gtg    688
Thr Glu Asn Arg Lys Leu Leu Gly Thr Val Ile Gln Tyr Gly Asn Val
105                 110                 115                 120
atc cag ctc ctg cat ttg aaa agc aat aaa tac ctg act gtg aat aag    736
Ile Gln Leu Leu His Leu Lys Ser Asn Lys Tyr Leu Thr Val Asn Lys
                125                 130                 135
agg ctc cca gcc ttg cta gag aag aat gcc atg agg gtg acg ttg gac    784
Arg Leu Pro Ala Leu Leu Glu Lys Asn Ala Met Arg Val Thr Leu Asp
            140                 145                 150
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gag gct gga aat gaa ggg tcc tgg ttt tac att caa cca ttc tac aag    832
Glu Ala Gly Asn Glu Gly Ser Trp Phe Tyr Ile Gln Pro Phe Tyr Lys
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ctt cgc tcc atc gga gac agt gtg gtc ata ggc gac aag gta gtt ttg    880
Leu Arg Ser Ile Gly Asp Ser Val Val Ile Gly Asp Lys Val Val Leu
    170                 175                 180
aat cct gtc aat gct ggc cag cct cta cat gcc agc agt cat cag ctg    928
Asn Pro Val Asn Ala Gly Gln Pro Leu His Ala Ser Ser His Gln Leu
185                 190                 195                 200
gtg gat aac cca ggc tgc aat gag gtc aac tcc gtc aac tgt aat aca    976
Val Asp Asn Pro Gly Cys Asn Glu Val Asn Ser Val Asn Cys Asn Thr
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agc tgg aag ata gtg ctt ttc atg aaa tgg agt gat aac aaa gac gac    1024
Ser Trp Lys Ile Val Leu Phe Met Lys Trp Ser Asp Asn Lys Asp Asp
            220                 225                 230
att ctc aaa gga ggt gat gtg gtg agg ctc ttc cat gcc gag caa gag    1072
Ile Leu Lys Gly Gly Asp Val Val Arg Leu Phe His Ala Glu Gln Glu
        235                 240                 245
aag ttt ctc acc tgt gat gag cac cgg aag aag cag cat gtg ttc ctg    1120
Lys Phe Leu Thr Cys Asp Glu His Arg Lys Lys Gln His Val Phe Leu
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agg acc acc ggc agg cag tca gcc acg tcg gcc acc agt tct aaa gcc    1168
Arg Thr Thr Gly Arg Gln Ser Ala Thr Ser Ala Thr Ser Ser Lys Ala
265                 270                 275                 280
ctg tgg gaa gtg gag gta gtc cag cac gac cca tgt cgg ggt gga gct    1216
Leu Trp Glu Val Glu Val Val Gln His Asp Pro Cys Arg Gly Gly Ala
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Gly Tyr Trp Asn Ser Leu Phe Arg Phe Lys His Leu Ala Thr Gly His
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Tyr Leu Ala Ala Glu Val Asp Pro Asp Phe Glu Glu Glu Cys Leu Glu

315 320 325

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Phe Gln Pro Ser Val Asp Pro Asp Gln Asp Ala Ser Arg Ser Arg Leu

330 335 340

aga aac gcg caa gaa aaa atg gta tac tct ctg gtc tcc gtg cct gaa 1408

Arg Asn Ala Gln Glu Lys Met Val Tyr Ser Leu Val Ser Val Pro Glu

345 350 355 360

ggc aac gac atc tcc tcc atc ttt gag cta gac ccc acg act ctg cgt 1456

Gly Asn Asp Ile Ser Ser Ile Phe Glu Leu Asp Pro Thr Thr Leu Arg

365 370 375

gga ggt gac agc ctt gtc cca agg aac tcc tat gtc cgt ctc aga cac 1504

Gly Gly Asp Ser Leu Val Pro Arg Asn Ser Tyr Val Arg Leu Arg His

380 385 390

ctg tgc acc aac acc tgg gta cac agc aca aac atc ccc atc gac aag 1552

Leu Cys Thr Asn Thr Trp Val His Ser Thr Asn Ile Pro Ile Asp Lys

395 400 405

gaa gag gag aag cct gtg atg ctg aaa att ggt acc tct ccc ctg aag 1600

Glu Glu Glu Lys Pro Val Met Leu Lys Ile Gly Thr Ser Pro Leu Lys

410 415 420

gag gac aag gaa gca ttt gcc ata gtt cct gtt tcc cct gct gag gtt 1648

Glu Asp Lys Glu Ala Phe Ala Ile Val Pro Val Ser Pro Ala Glu Val

425 430 435 440

cgg gac ctg gac ttt gcc aat gat gcc agc aag gtg ctg ggc tcc atc 1696

Arg Asp Leu Asp Phe Ala Asn Asp Ala Ser Lys Val Leu Gly Ser Ile

445 450 455

gct ggg aag ttg gaa aag ggc acc atc acc cag aat gag aga agg tct 1744

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Val Thr Lys Leu Leu Glu Asp Leu Val Tyr Phe Val Thr Gly Gly Thr
        475                     480                     485

aac tct ggc caa gac gtg ctt gaa gtt gtc ttc tct aag ccc aat cga    1840
Asn Ser Gly Gln Asp Val Leu Glu Val Val Phe Ser Lys Pro Asn Arg
    490                     495                     500

gag cgg cag aag ctg atg agg gaa cag aat att cto aag cag atc ttc    1888
Glu Arg Gln Lys Leu Met Arg Glu Gln Asn Ile Leu Lys Gln Ile Phe
505                     510                     515                 520

aag ctg ttg cag gcc ccc ttc acg gac tgc ggg gat ggc ccg atg ctt    1936
Lys Leu Leu Gln Ala Pro Phe Thr Asp Cys Gly Asp Gly Pro Met Leu
                525                     530                     535

cgg ctg gag gag ctg ggg gat cag cgc cat gct cct ttc aga cat att    1984
Arg Leu Glu Glu Leu Gly Asp Gln Arg His Ala Pro Phe Arg His Ile
            540                     545                     550

tgc cga ctc tgc tac agg gtc ctg cga cac tca cag caa gac tac agg    2032
Cys Arg Leu Cys Tyr Arg Val Leu Arg His Ser Gln Gln Asp Tyr Arg
        555                     560                     565

aag aac cag gag tac ata gcc aag cag ttt ggc ttc atg cag aag cag    2080
Lys Asn Gln Glu Tyr Ile Ala Lys Gln Phe Gly Phe Met Gln Lys Gln
    570                     575                     580

att ggc tat gac gtg ctg gcc gaa gac acc atc act gcc ctg ctc cac    2128
Ile Gly Tyr Asp Val Leu Ala Glu Asp Thr Ile Thr Ala Leu Leu His
585                     590                     595                 600

aac aac cgg aaa ctc ctg gag aag cac atc acc gcg gca gag att gac    2176
Asn Asn Arg Lys Leu Leu Glu Lys His Ile Thr Ala Ala Glu Ile Asp
                605                     610                     615
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620 625 630

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Tyr Leu Ser Asp Leu Cys Val Ser Met Asn Lys Ser Ile Pro Val Thr
635 640 645

cag gag ctc atc tgt aaa gct gtg ctc aat ccc acc aat gct gac atc 2320
Gln Glu Leu Ile Cys Lys Ala Val Leu Asn Pro Thr Asn Ala Asp Ile
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665 670 675 680

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Ser Thr Gly Glu Asn Ala Leu Glu Ala Gly Glu Asp Glu Glu Glu Val
685 690 695

tgg ctg ttc tgg agg gac agc aac aaa gag atc cgt agt aag agt gtc 2464
Trp Leu Phe Trp Arg Asp Ser Asn Lys Glu Ile Arg Ser Lys Ser Val
700 705 710

cgg gaa ttg gcg caa gat gct aaa gag gga cag aag gaa gac agg gac 2512
Arg Glu Leu Ala Gln Asp Ala Lys Glu Gly Gln Lys Glu Asp Arg Asp
715 720 725

atc ctc agc tac tac aga tat cag ctg aac ctc ttt gca agg atg tgt 2560
Ile Leu Ser Tyr Tyr Arg Tyr Gln Leu Asn Leu Phe Ala Arg Met Cys
730 735 740

ctg gac cgc cag tac ctg gcc atc aat gaa atc tcc ggg cag ctg gat 2608
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745 750 755 760

gtt gat ctc att ctc cgc tgc atg tct gac gag aac ctc ccc tac gac 2656
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765 770 775

ctc agg gca tcc ttt tgc cgc ctc atg ctt cac atg cat gtg gac cga 2704

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780 785 790

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795 800 805

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810 815 820

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825 830 835 840

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845 850 855

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860 865 870

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875 880 885

aag atc ctc ttg gca atc tta gac tgt gtc cat gtg acc act atc ttc 3040

Lys Ile Leu Leu Ala Ile Leu Asp Cys Val His Val Thr Thr Ile Phe

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Pro Ile Ser Lys Met Thr Lys Gly Glu Glu Asn Lys Gly Ser Asn Val

905 910 915 920

atg agg tct atc cat ggc gtt ggg gag ctg atg acc cag gtg gtg ctg 3136

Met Arg Ser Ile His Gly Val Gly Glu Leu Met Thr Gln Val Val Leu

925 930 935

cgg gga gga ggc ttc ttg ccc atg act ccc atg gct gcg gcc cct gaa 3184

Arg Gly Gly Gly Phe Leu Pro Met Thr Pro Met Ala Ala Ala Pro Glu

940 945 950

gga aat gtg aag cag gca gag cca gag aaa gag gac atc atg gtc atg 3232

Gly Asn Val Lys Gln Ala Glu Pro Glu Lys Glu Asp Ile Met Val Met

955 960 965

gac acc aag ttg aag atc att gaa ata ctc cag ttt att ttg aat gtg 3280

Asp Thr Lys Leu Lys Ile Ile Glu Ile Leu Gln Phe Ile Leu Asn Val

970 975 980

aga ttg gat tat agg atc tcc tgc ctc ctg tgt ata ttt aag cga gag 3328

Arg Leu Asp Tyr Arg Ile Ser Cys Leu Leu Cys Ile Phe Lys Arg Glu

985 990 995 1000

ttt gat gaa agc aat tcc cag tca tca gaa aca tcc tcc gga aac agc 3376

Phe Asp Glu Ser Asn Ser Gln Ser Ser Glu Thr Ser Ser Gly Asn Ser

1005 1010 1015

agc cag gaa ggg cca agt aat gtg cca ggt gct ctt gac ttt gaa cac 3424

Ser Gln Glu Gly Pro Ser Asn Val Pro Gly Ala Leu Asp Phe Glu His

1020 1025 1030

att gaa gaa caa gcg gaa ggc atc ttt gga gga agt gag gag aac aca 3472

Ile Glu Glu Gln Ala Glu Gly Ile Phe Gly Gly Ser Glu Glu Asn Thr

1035 1040 1045

cct ttg gac ctg gat gac cat ggt ggc aga acc ttc ctc agg gtc ctg 3520

Pro Leu Asp Leu Asp Asp His Gly Gly Arg Thr Phe Leu Arg Val Leu

1050 1055 1060

ctc cac ttg aca atg cat gac tac cca ccc ctg gtg tct ggg gcc ctg 3568

Leu His Leu Thr Met His Asp Tyr Pro Pro Leu Val Ser Gly Ala Leu

1065 1070 1075 1080

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Gln Leu Leu Phe Arg His Phe Ser Gln Arg Gln Glu Val Leu Gln Ala
1085 1090 1095

ttc aaa cag gtt caa ctg ctg gtt act agc caa gat gtg gac aac tac 3664
Phe Lys Gln Val Gln Leu Leu Val Thr Ser Gln Asp Val Asp Asn Tyr
1100 1105 1110

aaa cag atc aag caa gac ttg gac caa cta agg tcc att gtg gag aag 3712
Lys Gln Ile Lys Gln Asp Leu Asp Gln Leu Arg Ser Ile Val Glu Lys
1115 1120 1125

tct gag ctc tgg gtg tac aaa ggc caa ggt ccc gat gag cct atg gac 3760
Ser Glu Leu Trp Val Tyr Lys Gly Gln Gly Pro Asp Glu Pro Met Asp
1130 1135 1140

gga gcc tcc ggt gaa aat gag cat aag aaa acc gag gag ggg acg agc 3808
Gly Ala Ser Gly Glu Asn Glu His Lys Lys Thr Glu Glu Gly Thr Ser
1145 1150 1155 1160

aag cca ctg aag cac gag agc acc agc agc tac aac tac cga gtg gtg 3856
Lys Pro Leu Lys His Glu Ser Thr Ser Ser Tyr Asn Tyr Arg Val Val
1165 1170 1175

aaa gag att ttg att cga ctt agc aag ctc tgc gtg cag gag agc gcg 3904
Lys Glu Ile Leu Ile Arg Leu Ser Lys Leu Cys Val Gln Glu Ser Ala
1180 1185 1190

tcg gtg agg aag agc cgg aag cag cag caa cga ctg ctg agg aac atg 3952
Ser Val Arg Lys Ser Arg Lys Gln Gln Gln Arg Leu Leu Arg Asn Met
1195 1200 1205

ggc gca cac gct gtg gtg ctg gag ctg ctg cag atc ccc tac gag aag 4000
Gly Ala His Ala Val Val Leu Glu Leu Leu Gln Ile Pro Tyr Glu Lys
1210 1215 1220

gcc gaa gac aca aag atg caa gag atc atg cgg ctg gct cat gaa ttt 4048
Ala Glu Asp Thr Lys Met Gln Glu Ile Met Arg Leu Ala His Glu Phe

1225 1230 1235 1240

ttg cag aat ttc tgt gca ggc aac cag cag aat caa gct ttg ctg cat 4096

Leu Gln Asn Phe Cys Ala Gly Asn Gln Gln Asn Gln Ala Leu Leu His

1245 1250 1255

aaa cac ata aac ctg ttt ctc aag cca ggg atc ctg gag gca gtg acg 4144

Lys His Ile Asn Leu Phe Leu Lys Pro Gly Ile Leu Glu Ala Val Thr

1260 1265 1270

atg cag cac atc ttc atg aac aac ttc cag ctg tgc agt gag atc aac 4192

Met Gln His Ile Phe Met Asn Asn Phe Gln Leu Cys Ser Glu Ile Asn

1275 1280 1285

gag aga gtg gtc cag cac ttt gtt cac tgc ata gag acc cac ggt cga 4240

Glu Arg Val Val Gln His Phe Val His Cys Ile Glu Thr His Gly Arg

1290 1295 1300

aac gtc cag tat atc aag ttt ctc cag acg att gtc aag gca gaa ggg 4288

Asn Val Gln Tyr Ile Lys Phe Leu Gln Thr Ile Val Lys Ala Glu Gly

1305 1310 1315 1320

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Lys Phe Ile Lys Lys Cys Gln Asp Met Val Met Ala Glu Leu Val Asn

1325 1330 1335

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Ser Gly Glu Asp Val Leu Val Phe Tyr Asn Asp Arg Ala Ser Phe Gln

1340 1345 1350

act ctg atc cag atg atg cgg tcc gag cgt gac cgg atg gat gag aac 4432

Thr Leu Ile Gln Met Met Arg Ser Glu Arg Asp Arg Met Asp Glu Asn

1355 1360 1365

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Ser Pro Leu Met Tyr His Ile His Leu Val Glu Leu Leu Ala Val Cys

1370 1375 1380

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1385 1390 1395 1400

ccg ctc gat gac atc gtt cgt gtg gtc act cat gaa gac tgc atc ccc 4576
Pro Leu Asp Asp Ile Val Arg Val Val Thr His Glu Asp Cys Ile Pro
1405 1410 1415

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Glu Val Lys Ile Ala Tyr Ile Asn Phe Leu Asn His Cys Tyr Val Asp
1420 1425 1430

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Thr Glu Val Glu Met Lys Glu Ile Tyr Thr Ser Asn His Met Trp Lys
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1530 1535 1540

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Ser Arg Ala Ile Ala Ile Pro Val Asp Leu Asp Ser Gln Val Asn Asn

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Leu Phe Leu Lys Ser His Asn Ile Val Gln Lys Thr Ala Leu Asn Trp

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Arg Leu Ser Ala Arg Asn Ala Ala Arg Arg Asp Ser Val Leu Ala Ala

1580 1585 1590

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Ser Arg Asp Tyr Arg Asn Ile Ile Glu Arg Leu Gln Asp Ile Val Ser

1595 1600 1605

gcc cta gag gac cgg ctc agg ccc ctg gtg cag gct gag ctg tct gtg 5200

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1625 1630 1635 1640

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1645 1650 1655

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Lys His Thr Lys Gln Leu Leu Glu Glu Asn Glu Glu Lys Leu Cys Ile

1660 1665 1670

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1690 1695 1700

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1705 1710 1715 1720

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1725 1730 1735

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1740 1745 1750

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1755 1760 1765

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1770 1775 1780

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1785 1790 1795 1800

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1885 1890 1895

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1900 1905 1910

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1915 1920 1925

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1930 1935 1940

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1945 1950 1955 1960

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1995 2000 2005

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2010 2015 2020

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2025 2030 2035 2040

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Glu Tyr Cys Gln Gly Pro Cys His Glu Asn Gln Asn Cys Ile Ala Thr
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Val Glu Val Ile Lys Lys Ala Tyr Met Gln Gly Glu Val Glu Phe Glu
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2140 2145 2150

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His Asn Ile Tyr Ile Leu Ala His Gln Leu Ala Arg His Asn Lys Glu

2155 2160 2165

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2170 2175 2180

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2185 2190 2195 2200

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2220 2225 2230

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2235 2240 2245

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Tyr His Leu Leu Tyr Leu Leu Ile Cys Ala Met Gly Leu Phe Val His

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2650 2655 2660

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275 280 285

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290 295 300

Phe Lys His Leu Ala Thr Gly His Tyr Leu Ala Ala Glu Val Asp Pro

305 310 315 320

Asp Phe Glu Glu Glu Cys Leu Glu Phe Gln Pro Ser Val Asp Pro Asp

325 330 335

Gln Asp Ala Ser Arg Ser Arg Leu Arg Asn Ala Gln Glu Lys Met Val

340 345 350

Tyr Ser Leu Val Ser Val Pro Glu Gly Asn Asp Ile Ser Ser Ile Phe

355 360 365

Glu Leu Asp Pro Thr Thr Leu Arg Gly Gly Asp Ser Leu Val Pro Arg

370 375 380

Asn Ser Tyr Val Arg Leu Arg His Leu Cys Thr Asn Thr Trp Val His

385 390 395 400

Ser Thr Asn Ile Pro Ile Asp Lys Glu Glu Glu Lys Pro Val Met Leu

405 410 415

Lys Ile Gly Thr Ser Pro Leu Lys Glu Asp Lys Glu Ala Phe Ala Ile

420 425 430

Val Pro Val Ser Pro Ala Glu Val Arg Asp Leu Asp Phe Ala Asn Asp

435 440 445

Ala Ser Lys Val Leu Gly Ser Ile Ala Gly Lys Leu Glu Lys Gly Thr

450 455 460

Ile Thr Gln Asn Glu Arg Arg Ser Val Thr Lys Leu Leu Glu Asp Leu

465 470 475 480

Val Tyr Phe Val Thr Gly Gly Thr Asn Ser Gly Gln Asp Val Leu Glu

485 490 495

Val Val Phe Ser Lys Pro Asn Arg Glu Arg Gln Lys Leu Met Arg Glu
500 505 510

Gln Asn Ile Leu Lys Gln Ile Phe Lys Leu Leu Gln Ala Pro Phe Thr
515 520 525

Asp Cys Gly Asp Gly Pro Met Leu Arg Leu Glu Glu Leu Gly Asp Gln
530 535 540

Arg His Ala Pro Phe Arg His Ile Cys Arg Leu Cys Tyr Arg Val Leu
545 550 555 560

Arg His Ser Gln Gln Asp Tyr Arg Lys Asn Gln Glu Tyr Ile Ala Lys
565 570 575

Gln Phe Gly Phe Met Gln Lys Gln Ile Gly Tyr Asp Val Leu Ala Glu
580 585 590

Asp Thr Ile Thr Ala Leu Leu His Asn Asn Arg Lys Leu Leu Glu Lys
595 600 605

His Ile Thr Ala Ala Glu Ile Asp Thr Phe Val Ser Leu Val Arg Lys
610 615 620

Asn Arg Glu Pro Arg Phe Leu Asp Tyr Leu Ser Asp Leu Cys Val Ser
625 630 635 640

Met Asn Lys Ser Ile Pro Val Thr Gln Glu Leu Ile Cys Lys Ala Val
645 650 655

Leu Asn Pro Thr Asn Ala Asp Ile Leu Ile Glu Thr Lys Leu Val Leu
660 665 670

Ser Arg Phe Glu Phe Glu Gly Val Ser Thr Gly Glu Asn Ala Leu Glu
675 680 685

Ala Gly Glu Asp Glu Glu Glu Val Trp Leu Phe Trp Arg Asp Ser Asn
690 695 700

Lys Glu Ile Arg Ser Lys Ser Val Arg Glu Leu Ala Gln Asp Ala Lys
705 710 715 720

Glu Gly Gln Lys Glu Asp Arg Asp Ile Leu Ser Tyr Tyr Arg Tyr Gln

725 730 735

Leu Asn Leu Phe Ala Arg Met Cys Leu Asp Arg Gln Tyr Leu Ala Ile

740 745 750

Asn Glu Ile Ser Gly Gln Leu Asp Val Asp Leu Ile Leu Arg Cys Met

755 760 765

Ser Asp Glu Asn Leu Pro Tyr Asp Leu Arg Ala Ser Phe Cys Arg Leu

770 775 780

Met Leu His Met His Val Asp Arg Asp Pro Gln Glu Gln Val Thr Pro

785 790 795 800

Val Lys Tyr Ala Arg Leu Trp Ser Glu Ile Pro Ser Glu Ile Ala Ile

805 810 815

Asp Asp Tyr Asp Ser Ser Gly Thr Ser Lys Asp Glu Ile Lys Glu Arg

820 825 830

Phe Ala Gln Thr Met Glu Phe Val Glu Glu Tyr Leu Arg Asp Val Val

835 840 845

Cys Gln Arg Phe Pro Phe Ser Asp Lys Glu Lys Asn Lys Leu Thr Phe

850 855 860

Glu Val Val Asn Leu Ala Arg Asn Leu Ile Tyr Phe Gly Phe Tyr Asn

865 870 875 880

Phe Ser Asp Leu Leu Arg Leu Thr Lys Ile Leu Leu Ala Ile Leu Asp

885 890 895

Cys Val His Val Thr Thr Ile Phe Pro Ile Ser Lys Met Thr Lys Gly

900 905 910

Glu Glu Asn Lys Gly Ser Asn Val Met Arg Ser Ile His Gly Val Gly

915 920 925

Glu Leu Met Thr Gln Val Val Leu Arg Gly Gly Gly Phe Leu Pro Met

930 935 940

Thr Pro Met Ala Ala Ala Pro Glu Gly Asn Val Lys Gln Ala Glu Pro

945 950 955 960

Glu Lys Glu Asp Ile Met Val Met Asp Thr Lys Leu Lys Ile Ile Glu

965 970 975

Ile Leu Gln Phe Ile Leu Asn Val Arg Leu Asp Tyr Arg Ile Ser Cys

980 985 990

Leu Leu Cys Ile Phe Lys Arg Glu Phe Asp Glu Ser Asn Ser Gln Ser

995 1000 1005

Ser Glu Thr Ser Ser Gly Asn Ser Ser Gln Glu Gly Pro Ser Asn Val

1010 1015 1020

Pro Gly Ala Leu Asp Phe Glu His Ile Glu Glu Gln Ala Glu Gly Ile

025 1030 1035 1040

Phe Gly Gly Ser Glu Glu Asn Thr Pro Leu Asp Leu Asp Asp His Gly

1045 1050 1055

Gly Arg Thr Phe Leu Arg Val Leu Leu His Leu Thr Met His Asp Tyr

1060 1065 1070

Pro Pro Leu Val Ser Gly Ala Leu Gln Leu Leu Phe Arg His Phe Ser

1075 1080 1085

Gln Arg Gln Glu Val Leu Gln Ala Phe Lys Gln Val Gln Leu Leu Val

1090 1095 1100

Thr Ser Gln Asp Val Asp Asn Tyr Lys Gln Ile Lys Gln Asp Leu Asp

105 1110 1115 1120

Gln Leu Arg Ser Ile Val Glu Lys Ser Glu Leu Trp Val Tyr Lys Gly

1125 1130 1135

Gln Gly Pro Asp Glu Pro Met Asp Gly Ala Ser Gly Glu Asn Glu His

1140 1145 1150

Lys Lys Thr Glu Glu Gly Thr Ser Lys Pro Leu Lys His Glu Ser Thr

1155 1160 1165

Ser Ser Tyr Asn Tyr Arg Val Val Lys Glu Ile Leu Ile Arg Leu Ser

1170 1175 1180

Lys Leu Cys Val Gln Glu Ser Ala Ser Val Arg Lys Ser Arg Lys Gln

185 1190 1195 1200

Gln Gln Arg Leu Leu Arg Asn Met Gly Ala His Ala Val Val Leu Glu

1205 1210 1215

Leu Leu Gln Ile Pro Tyr Glu Lys Ala Glu Asp Thr Lys Met Gln Glu

1220 1225 1230

Ile Met Arg Leu Ala His Glu Phe Leu Gln Asn Phe Cys Ala Gly Asn

1235 1240 1245

Gln Gln Asn Gln Ala Leu Leu His Lys His Ile Asn Leu Phe Leu Lys

1250 1255 1260

Pro Gly Ile Leu Glu Ala Val Thr Met Gln His Ile Phe Met Asn Asn

265 1270 1275 1280

Phe Gln Leu Cys Ser Glu Ile Asn Glu Arg Val Val Gln His Phe Val

1285 1290 1295

His Cys Ile Glu Thr His Gly Arg Asn Val Gln Tyr Ile Lys Phe Leu

1300 1305 1310

Gln Thr Ile Val Lys Ala Glu Gly Lys Phe Ile Lys Lys Cys Gln Asp

1315 1320 1325

Met Val Met Ala Glu Leu Val Asn Ser Gly Glu Asp Val Leu Val Phe

1330 1335 1340

Tyr Asn Asp Arg Ala Ser Phe Gln Thr Leu Ile Gln Met Met Arg Ser

345 1350 1355 1360

Glu Arg Asp Arg Met Asp Glu Asn Ser Pro Leu Met Tyr His Ile His

1365 1370 1375

Leu Val Glu Leu Leu Ala Val Cys Thr Glu Gly Lys Asn Val Tyr Thr

1380 1385 1390

Glu Ile Lys Cys Asn Ser Leu Leu Pro Leu Asp Asp Ile Val Arg Val

1395 1400 1405

Val Thr His Glu Asp Cys Ile Pro Glu Val Lys Ile Ala Tyr Ile Asn

1410 1415 1420

Phe Leu Asn His Cys Tyr Val Asp Thr Glu Val Glu Met Lys Glu Ile

425 1430 1435 1440

Tyr Thr Ser Asn His Met Trp Lys Leu Phe Glu Asn Phe Leu Val Asp

1445 1450 1455

Ile Cys Arg Ala Cys Asn Asn Thr Ser Asp Arg Lys His Ala Asp Ser

1460 1465 1470

Ile Leu Glu Lys Tyr Val Thr Glu Ile Val Met Ser Ile Val Thr Thr

1475 1480 1485

Phe Phe Ser Ser Pro Phe Ser Asp Gln Ser Thr Thr Leu Gln Thr Arg

1490 1495 1500

Gln Pro Val Phe Val Gln Leu Leu Gln Gly Val Phe Arg Val Tyr His

505 1510 1515 1520

Cys Asn Trp Leu Met Pro Ser Gln Lys Ala Ser Val Glu Ser Cys Ile

1525 1530 1535

Arg Val Leu Ser Asp Val Ala Lys Ser Arg Ala Ile Ala Ile Pro Val

1540 1545 1550

Asp Leu Asp Ser Gln Val Asn Asn Leu Phe Leu Lys Ser His Asn Ile

1555 1560 1565

Val Gln Lys Thr Ala Leu Asn Trp Arg Leu Ser Ala Arg Asn Ala Ala

1570 1575 1580

Arg Arg Asp Ser Val Leu Ala Ala Ser Arg Asp Tyr Arg Asn Ile Ile

585 1590 1595 1600

Glu Arg Leu Gln Asp Ile Val Ser Ala Leu Glu Asp Arg Leu Arg Pro

1605 1610 1615

Leu Val Gln Ala Glu Leu Ser Val Leu Val Asp Val Leu His Arg Pro

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Glu Leu Leu Phe Pro Glu Asn Thr Asp Ala Arg Arg Lys Cys Glu Ser

1635 1640 1645

Gly Gly Phe Ile Cys Lys Leu Ile Lys His Thr Lys Gln Leu Leu Glu

1650 1655 1660

Glu Asn Glu Glu Lys Leu Cys Ile Lys Val Leu Gln Thr Leu Arg Glu

665 1670 1675 1680

Met Met Thr Lys Asp Arg Gly Tyr Gly Glu Lys Gln Ile Ser Ile Asp

1685 1690 1695

Glu Ser Glu Asn Ala Glu Leu Pro Gln Ala Pro Glu Ala Glu Asn Ser

1700 1705 1710

Thr Glu Gln Glu Leu Glu Pro Ser Pro Pro Leu Arg Gln Leu Glu Asp

1715 1720 1725

His Lys Arg Gly Glu Ala Leu Arg Gln Ile Leu Val Asn Arg Tyr Tyr

1730 1735 1740

Gly Asn Ile Arg Pro Ser Gly Arg Arg Glu Ser Leu Thr Ser Phe Gly

745 1750 1755 1760

Asn Gly Pro Leu Ser Pro Gly Gly Pro Ser Lys Pro Gly Gly Gly Gly

1765 1770 1775

Gly Gly Pro Gly Ser Ser Ser Thr Ser Arg Gly Glu Met Ser Leu Ala

1780 1785 1790

Glu Val Gln Cys His Leu Asp Lys Glu Gly Ala Ser Asn Leu Val Ile

1795 1800 1805

Asp Leu Ile Met Asn Ala Ser Ser Asp Arg Val Phe His Glu Ser Ile

1810 1815 1820

Leu Leu Ala Ile Ala Leu Leu Glu Gly Gly Asn Thr Thr Ile Gln His

825 1830 1835 1840

Ser Phe Phe Cys Arg Leu Thr Glu Asp Lys Lys Ser Glu Lys Phe Phe

1845 1850 1855

Lys Val Phe Tyr Asp Arg Met Lys Val Ala Gln Gln Glu Ile Lys Ala

1860 1865 1870

Thr Val Thr Val Asn Thr Ser Asp Leu Gly Asn Lys Lys Lys Asp Asp

1875 1880 1885

Glu Val Asp Arg Asp Ala Pro Ser Arg Lys Lys Ala Lys Glu Pro Thr

1890 1895 1900

Thr Gln Ile Thr Glu Glu Val Arg Asp Gln Leu Leu Glu Ala Ser Ala

905 1910 1915 1920

Ala Thr Arg Lys Ala Phe Thr Thr Phe Arg Arg Glu Ala Asp Pro Asp

1925 1930 1935

Asp His Tyr Gln Ser Gly Glu Gly Thr Gln Ala Thr Thr Asp Lys Ala

1940 1945 1950

Lys Asp Asp Leu Glu Met Ser Ala Val Ile Thr Ile Met Gln Pro Ile

1955 1960 1965

Leu Arg Phe Leu Gln Leu Leu Cys Glu Asn His Asn Arg Asp Leu Gln

1970 1975 1980

Asn Phe Leu Arg Cys Gln Asn Asn Lys Thr Asn Tyr Asn Leu Val Cys

985 1990 1995 2000

Glu Thr Leu Gln Phe Leu Asp Cys Ile Cys Gly Ser Thr Thr Gly Gly

2005 2010 2015

Leu Gly Leu Leu Gly Leu Tyr Ile Asn Glu Lys Asn Val Ala Leu Ile

2020 2025 2030

Asn Gln Thr Leu Glu Ser Leu Thr Glu Tyr Cys Gln Gly Pro Cys His

2035 2040 2045

Glu Asn Gln Asn Cys Ile Ala Thr His Glu Ser Asn Gly Ile Asp Ile

2050 2055 2060

Ile Thr Ala Leu Ile Leu Asn Asp Ile Asn Pro Leu Gly Lys Lys Arg

065 2070 2075 2080

Met Asp Leu Val Leu Glu Leu Lys Asn Asn Ala Ser Lys Leu Leu Leu

2085 2090 2095

Ala Ile Met Glu Ser Arg His Asp Ser Glu Asn Ala Glu Arg Ile Leu

2100 2105 2110

Tyr Asn Met Arg Pro Lys Glu Leu Val Glu Val Ile Lys Lys Ala Tyr

2115 2120 2125

Met Gln Gly Glu Val Glu Phe Glu Asp Gly Glu Asn Gly Glu Asp Gly

2130 2135 2140

Ala Ala Ser Pro Arg Asn Val Gly His Asn Ile Tyr Ile Leu Ala His

145 2150 2155 2160

Gln Leu Ala Arg His Asn Lys Glu Leu Gln Thr Met Leu Lys Pro Gly

2165 2170 2175

Gly Gln Val Asp Gly Asp Glu Ala Leu Glu Phe Tyr Ala Lys His Thr

2180 2185 2190

Ala Gln Ile Glu Ile Val Arg Leu Asp Arg Thr Met Glu Gln Ile Val

2195 2200 2205

Phe Pro Val Pro Ser Ile Cys Glu Phe Leu Thr Lys Glu Ser Lys Leu

2210 2215 2220

Arg Ile Tyr Tyr Thr Thr Glu Arg Asp Glu Gln Gly Ser Lys Ile Asn

225 2230 2235 2240

Asp Phe Phe Leu Arg Ser Glu Asp Leu Phe Asn Glu Met Asn Trp Gln

2245 2250 2255

Lys Lys Leu Arg Ala Gln Pro Val Leu Tyr Trp Cys Ala Arg Asn Met

2260 2265 2270

Ser Phe Trp Ser Ser Ile Ser Phe Asn Leu Ala Val Leu Met Asn Leu

2275 2280 2285

Leu Val Ala Phe Phe Tyr Pro Phe Lys Gly Val Arg Gly Gly Thr Leu

2290 2295 2300

Glu Pro His Trp Ser Gly Leu Leu Trp Thr Ala Met Leu Ile Ser Leu

305 2310 2315 2320

Ala Ile Val Ile Ala Leu Pro Lys Pro His Gly Ile Arg Ala Leu Ile

2325 2330 2335

Ala Ser Thr Ile Leu Arg Leu Ile Phe Ser Val Gly Leu Gln Pro Thr

2340 2345 2350

Leu Phe Leu Leu Gly Ala Phe Asn Val Cys Asn Lys Ile Ile Phe Leu

2355 2360 2365

Met Ser Phe Val Gly Asn Cys Gly Thr Phe Thr Arg Gly Tyr Arg Ala

2370 2375 2380

Met Val Leu Asp Val Glu Phe Leu Tyr His Leu Leu Tyr Leu Leu Ile

385 2390 2395 2400

Cys Ala Met Gly Leu Phe Val His Glu Phe Phe Tyr Ser Leu Leu Leu

2405 2410 2415

Phe Asp Leu Val Tyr Arg Glu Glu Thr Leu Leu Asn Val Ile Lys Ser

2420 2425 2430

Val Thr Arg Asn Gly Arg Ser Ile Ile Leu Thr Ala Val Leu Ala Leu

2435 2440 2445

Ile Leu Val Tyr Leu Phe Ser Ile Val Gly Tyr Leu Phe Phe Lys Asp

2450 2455 2460

Asp Phe Ile Leu Glu Val Asp Arg Leu Pro Asn Glu Thr Ala Val Pro

465 2470 2475 2480

Glu Thr Gly Glu Ser Leu Ala Asn Asp Phe Leu Tyr Ser Asp Val Cys

2485 2490 2495

Arg Val Glu Thr Gly Glu Asn Cys Thr Ser Pro Ala Pro Lys Glu Glu

2500 2505 2510

Leu Leu Pro Ala Glu Glu Thr Glu Gln Asp Lys Glu His Thr Cys Glu

2515 2520 2525

Thr Leu Leu Met Cys Ile Val Thr Val Leu Ser His Gly Leu Arg Ser

2530 2535 2540

Gly Gly Gly Val Gly Asp Val Leu Arg Lys Pro Ser Lys Glu Glu Pro

545 2550 2555 2560

Leu Phe Ala Ala Arg Val Ile Tyr Asp Leu Leu Phe Phe Phe Met Val

2565 2570 2575

Ile Ile Ile Val Leu Asn Leu Ile Phe Gly Val Ile Ile Asp Thr Phe

2580 2585 2590

Ala Asp Leu Arg Ser Glu Lys Gln Lys Lys Glu Glu Ile Leu Lys Thr

2595 2600 2605

Thr Cys Phe Ile Cys Gly Leu Glu Arg Asp Lys Phe Asp Asn Lys Thr

2610 2615 2620

Val Thr Phe Glu Glu His Ile Lys Glu Glu His Asn Met Trp His Tyr

625 2630 2635 2640

Leu Cys Phe Ile Val Leu Val Lys Val Lys Asp Ser Thr Glu Tyr Thr

2645 2650 2655

Gly Pro Glu Ser Tyr Val Ala Glu Met Ile Arg Glu Arg Asn Leu Asp

2660 2665 2670

Trp Phe Leu Arg Met Arg Ala Met Ser Leu Val Ser Ser Asp Ser Glu

2675 2680 2685

Gly Glu Gln Asn Glu Leu Arg Asn Leu Gln Glu Lys Leu Glu Ser Thr

2690 2695 2700

Met Lys Leu Val Thr Asn Leu Ser Gly Gln Leu Ser Glu Leu Lys Asp

705 2710 2715 2720

Gln Met Thr Glu Gln Arg Lys Gln Lys Gln Arg Ile Gly Leu Leu Gly

2725 2730 2735

His Pro Pro His Met Asn Val Asn Pro Gln Gln Pro Ala

2740 2745

<210> 5

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Synthetic DNA

<400> 5

tgtcagacat atgcgtgttg gaa 23

<210> 6

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Synthetic DNA

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cgcgggatcc ttatttccgg ttgttgtgga gcaggg 36

<210> 7

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Synthetic DNA

<400> 7

ccggaattct tatttccggt tgttgtggag caggg 35

<210> 8

<211> 37

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Synthetic DNA

<400> 8

cgcggatcca tgaaatggag tgataacaaa gacgaca 37

<210> 9

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Synthetic DNA

<400> 9

gagagcggca ggcactgatg aggg 24

<210> 10

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Synthetic DNA

<400> 10

ccctcatcag tgcctgccgc tctc 24

<210> 11

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Synthetic DNA

<400> 11

gctgaggttc aagacctgga ctttg 25

<210> 12

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Synthetic DNA

<400> 12

aaagtccagg tcttgaacct cagc 24

Claims

1. A recombinant polypeptide of the following (a), (b) or (c):

   (a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO: 2;
   (b) a polypeptide comprising an amino acid sequence having deletion, substitution or addition of at least one amino acid in the amino acid sequence shown in SEQ ID NO: 2, and having a high affinity binding activity to inositol 1,4,5-trisphosphate; or
   (c) a polypeptide having at least 70% homology with the amino acid sequence shown in SEQ ID NO: 2, and having a high affinity binding activity with inositol 1,4,5-trisphosphate.

2. A gene coding for a polypeptide of the following (a), (b) or (c):

   (a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO: 2;
   (b) a polypeptide comprising an amino acid sequence having deletion, substitution or addition of at least one amino acid in the amino acid sequence shown in SEQ ID NO: 2, and having a high affinity binding activity with inositol 1,4,5-trisphosphate; or
   (c) a polypeptide having at least 70% homology with the amino acid sequence shown in SEQ ID NO: 2, and having a high affinity binding activity to inositol 1,4,5-trisphosphate.

3. A gene comprising DNA of the following (d) or (e):

   (d) DNA of a nucleotide sequence shown in SEQ ID NO: 1; or
   (e) DNA of a nucleotide sequence having at least 70% homology with the DNA of the nucleotide sequence shown in SEQ ID NO: 1, and coding for a polypeptide having a high affinity binding activity with inositol 1,4,5-trisphosphate.

4. A gene comprising DNA which has a nucleotide sequence having at least 70% homology with the gene of claim 2, and which codes for a polypeptide having a high affinity binding activity with inositol 1,4,5-trisphosphate.

5. A recombinant vector comprising the gene of any one of claims 2 to 4.

6. A transformant comprising the recombinant vector of claim 5.

7. A method for producing the polypeptide of any one of the preceding claims, comprising:

   culturing the transformant of claim 6; and
   collecting, from the obtained culture, a polypeptide having a high affinity binding activity to inositol 1,4,5-trisphosphate.

8. An antagonist for IP_3-induced calcium comprising the protein of claim 1.

9. A therapeutic agent diseases associated with calcium production comprising the protein of claim 1.

10. An agent for gene therapy for diseases associated with calcium production comprising the gene of claims 2 or 3.

11. The therapeutic agent of claims 10 or 11, wherein the disease is at least one disease selected from the group consisting of diseases in the nervous system, blood vascular system, respiratory system, digestive system, lymphatic system, urinary system and reproduction system.

FIG. 1

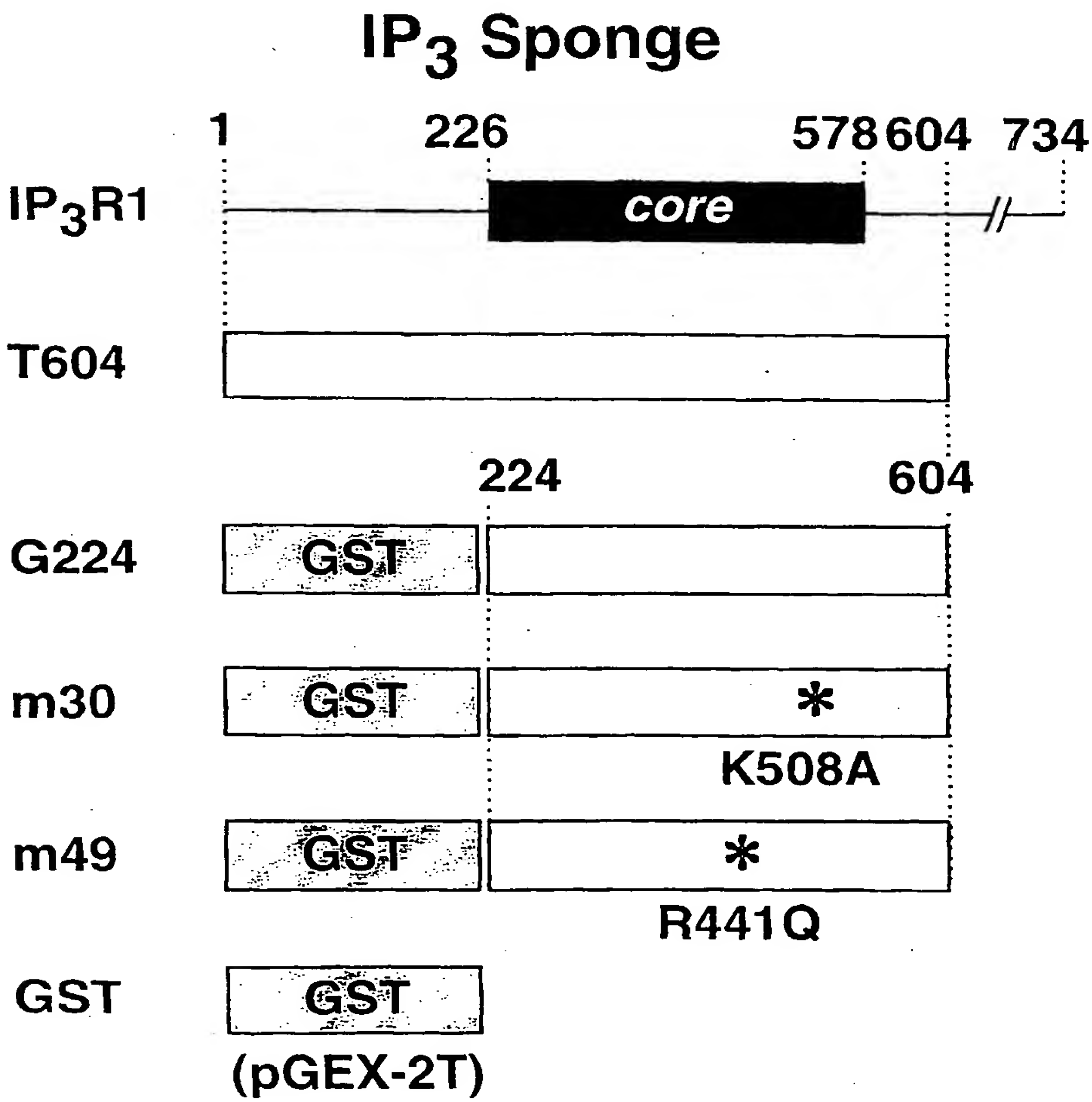

IP3 Sponge
1
226
578
604
734
IP3R1
core
T604
224
604
G224
GST
m30
GST
*
K508A
m49
GST
*
R441Q
GST
GST
(pGEX-2T)

FIG. 2A

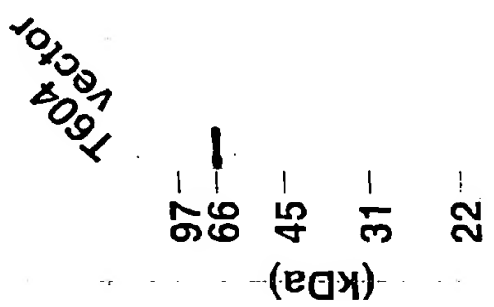


FIG. 2B

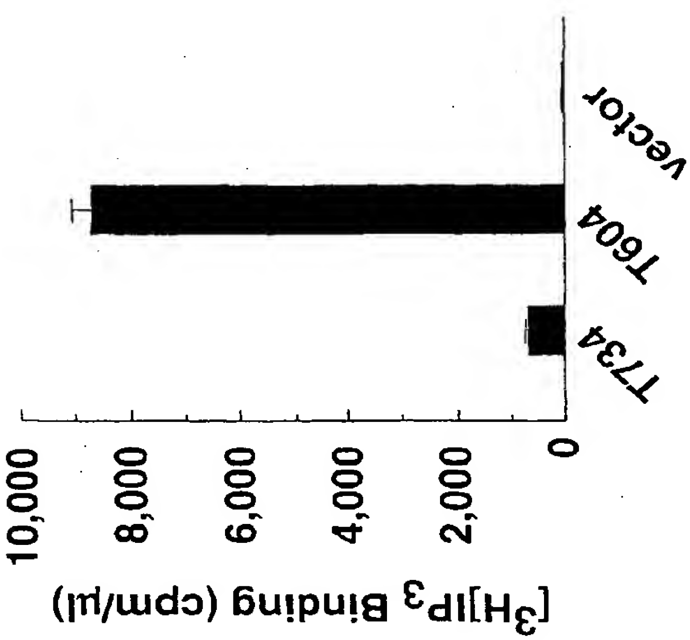


FIG. 2C

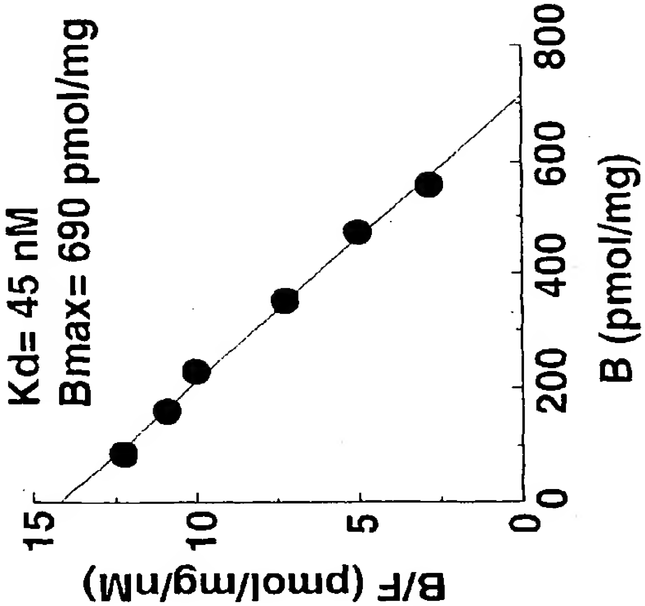


FIG. 3A

G224

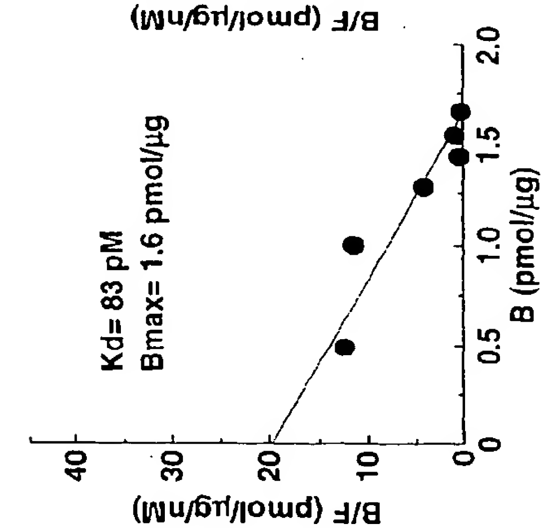


FIG. 3B

G224m49

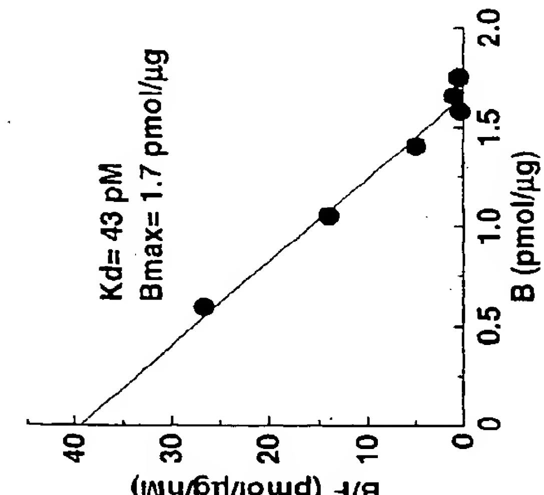


FIG. 3C

G224m30

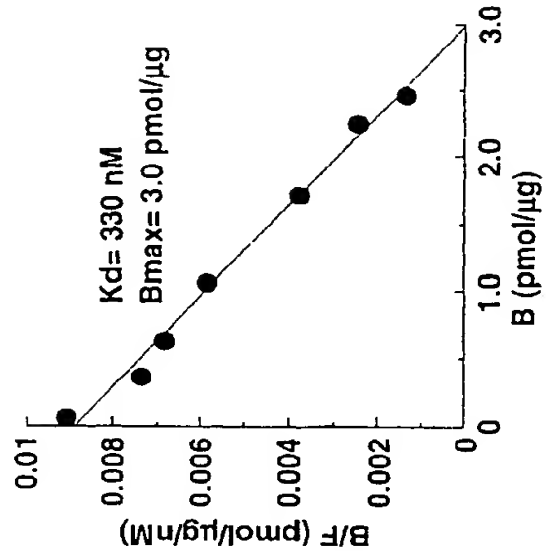


FIG. 4

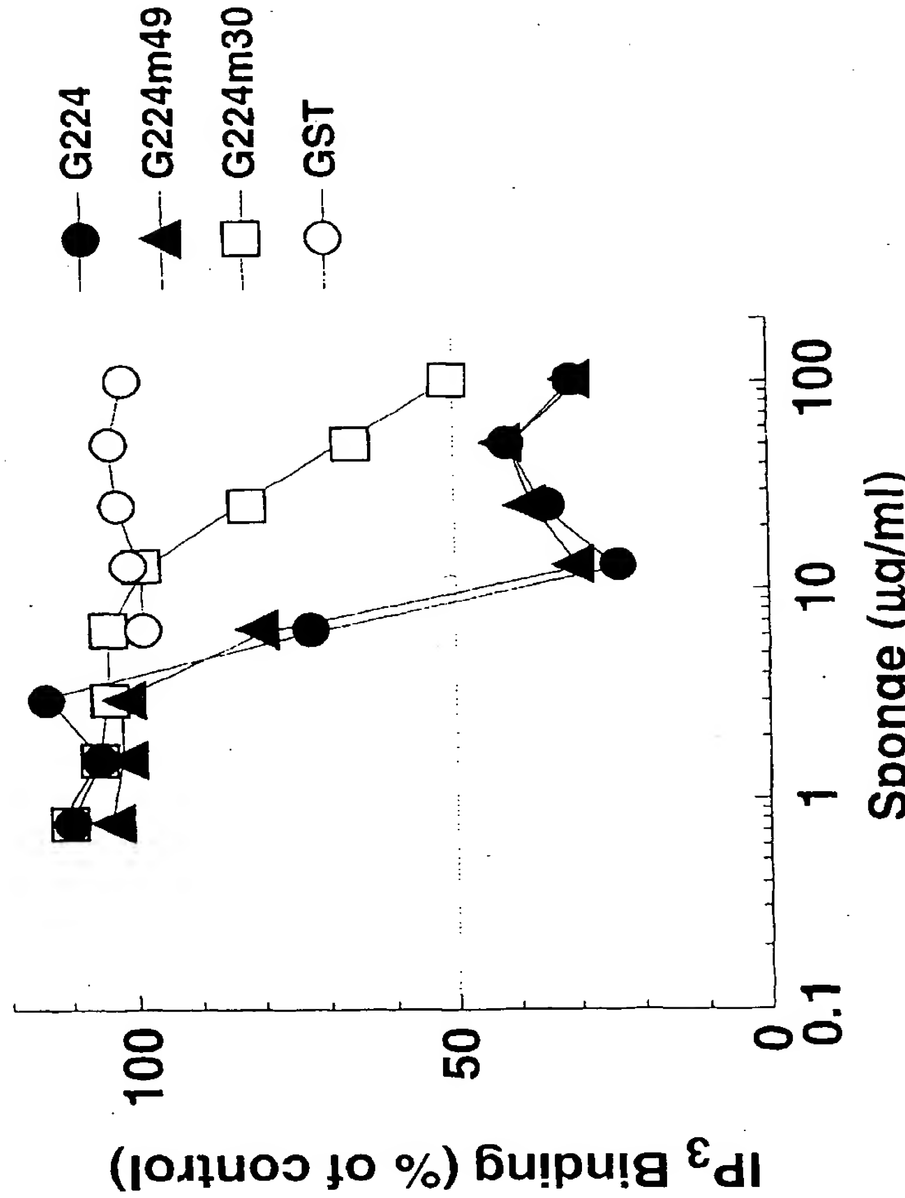

IP3 Binding (% of control)
100
50
0
0.1
1
10
100
Sponge (µg/ml)
G224
G224m49
G224m30
GST

FIG. 5A

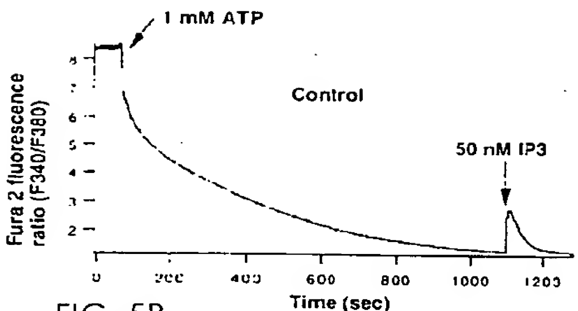


FIG. 5B

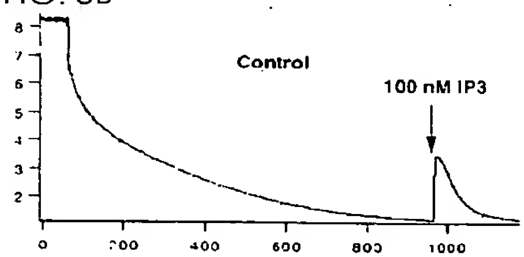


FIG. 5C

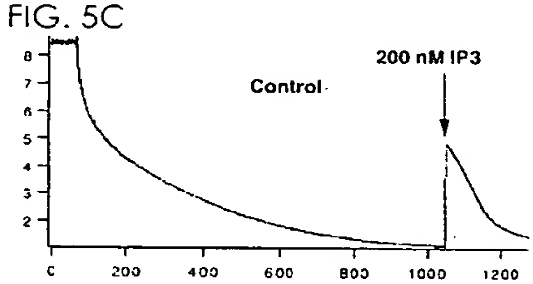


FIG. 5D

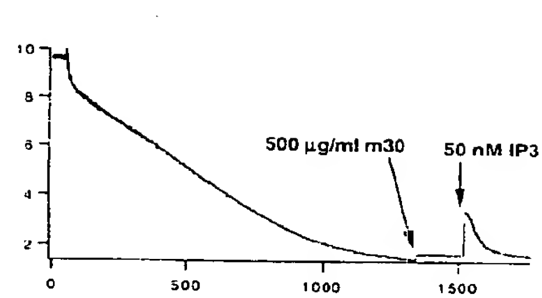


FIG. 5E

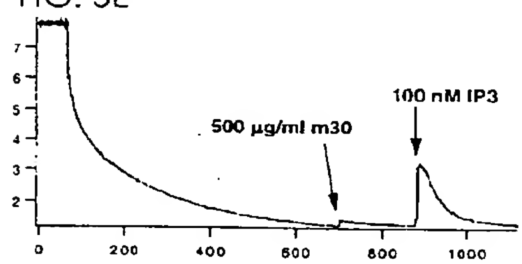


FIG. 5F

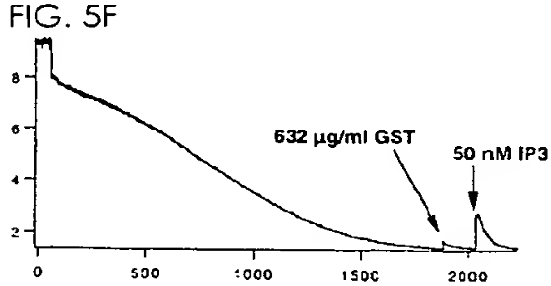


FIG. 6A

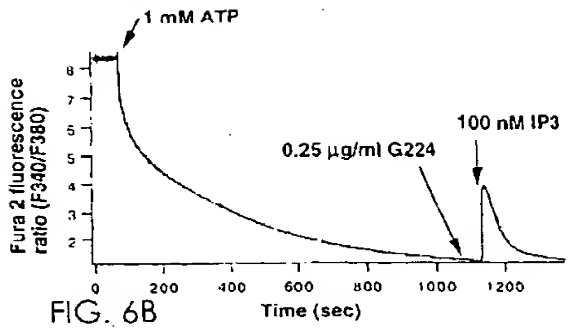


FIG. 6B

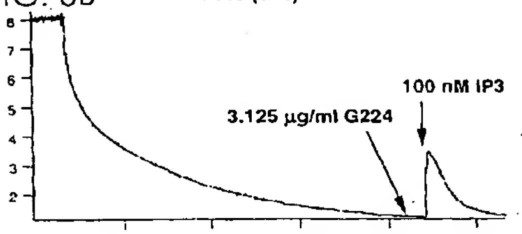


FIG. 6C

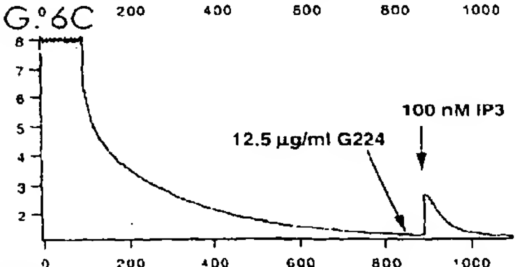


FIG. 6D

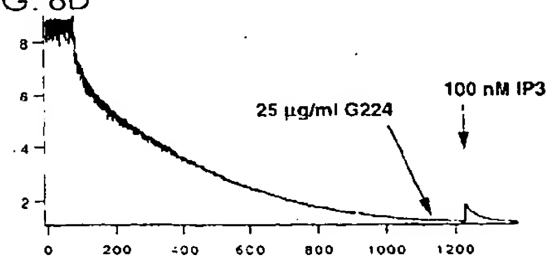


FIG. 6E

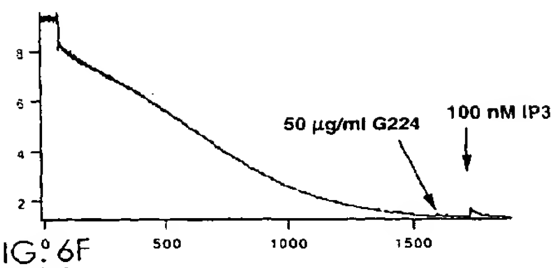


FIG. 6F

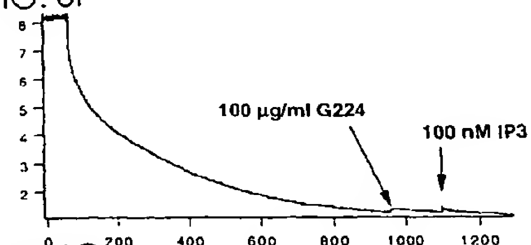


FIG. 6G

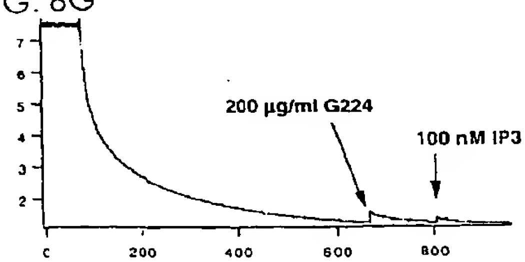
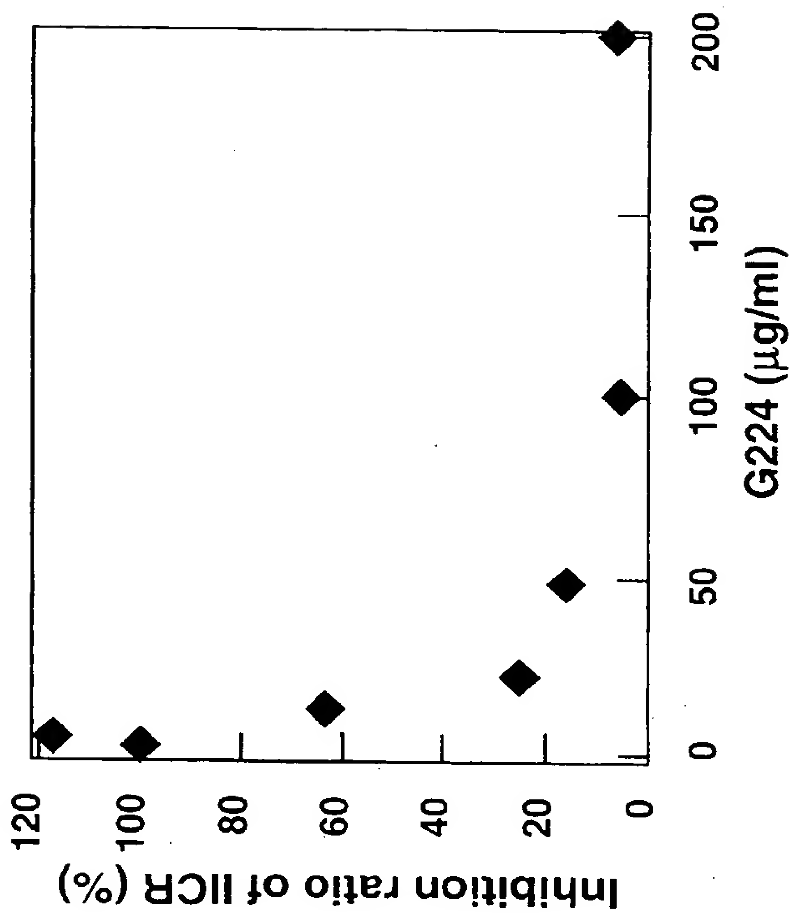


FIG. 7



Inhibition ratio of IICR (%)
120
100
80
60
40
20
0
G224 (µg/ml)
0
50
100
150
200

(19) Europäisches Patentamt
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(71) Applicants:
- Riken
Wako-shi, Saitama 351-0198 (JP)
- Mikoshiba, Katsuhiko
Tokyo 181-0001 (JP)

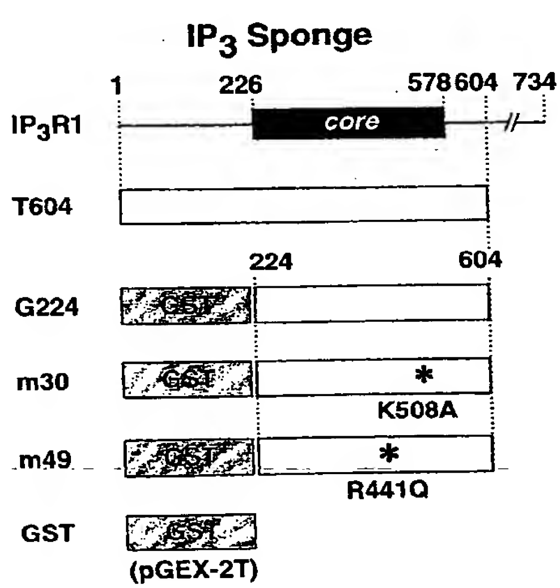
(72) Inventors:
- Mikoshiba, Katsuhiko
Mitaka-shi, Tokyo 181-0001 (JP)
- Furuichi, Teiichi
Chiba-shi, Chiba 263-0022 (JP)
- Yoshikawa, Fumio
Yokohama-shi, Kanagawa 234-0054 (JP)
- Uchiyama, Tsuyoshi
Shinagawa-ku, Tokyo 141-0021 (JP)

(74) Representative: Maschio, Antonio et al
D Young & Co,
21 New Fetter Lane
London EC4A 1DA (GB)

(54) **High affinity IP3-binding polypeptide**

(57) The present invention provides a high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate, to a gene encoding the polypeptide, to a recombinant vector including the gene, to a transformant including the vector and to a method for producing the high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate.

FIG. 1



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EUROPEAN SEARCH REPORT

Application Number
EP 99 30 6879

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	FURUICHI T ET AL.: "Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P400" NATURE, vol. 342, 2 November 1989 (1989-11-02), pages 32-38, XP002141737 * the whole document *	1-7	C12N15/12 C07K14/705 C12N1/20 C12N15/62 A61K38/17 A61K48/00
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X	DATABASE WPI Week 9648 Derwent Publications Ltd., London, GB; AN 1996-482258 XP002141738 "Human type I tri-phosphate receptor" & JP 08 245698 A (SOSEI KK), 24 September 1996 (1996-09-24) * abstract *	1-9	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	4 July 2000	Oderwald, H

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